UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE ODONTOLOGÍA

Departamento de Estomatología III (Medicina y Cirugía Bucofacial)

TESIS DOCTORAL

Modelo de biofilm bacteriano *in vitro*: evaluación en superficies de implantes y comparación de agentes microbianos

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Director

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Madrid, 2018

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Modelo de biofilm bacteriano *in vitro*: evaluación en superficies de implantes y comparación de agentes antimicrobianos.

Memoria presentada para optar al grado de Doctor por

Dña. Eva Fernández Vidal

Director

Prof. Dr. David Herrera González

Madrid, 2017

A mi marido Nacho, mi hija Bárbara y a mis padres
Andrés y Tana por darme ánimo todos los días.

Veritas in simplice
(La verdad está en lo sencillo)
Carlos Dómine

Al comenzar el programa de Doctorado creía que consistía en un proyecto personal, en el que el resultado dependía de mí y de mi esfuerzo. Pero en realidad es un proyecto en el que se ven involucradas muchas personas, familia, amigos y compañeros. Hoy no estaría escribiendo esta tesis si mi Director David Herrera no me hubiera aceptado como doctorando, hace ya 5 años, por lo que, sin duda, es la primera persona a la que tengo que agradecer estar hoy aquí y no sólo por ese motivo, también por haber estado disponible siempre que lo necesité y por corregir tan rápidamente todo lo que le enviaba, pese a estar ocupadísimo.

Todas y cada una de las profesionales del laboratorio de microbiología me ayudaron a moverme por el laboratorio, no sin esfuerzo, ya que acostumbrada al ámbito clínico, todo era nuevo para mí. Mil gracias a todas: María José Marín, Elena Figuero, Itziar González, Ana O´Connor y Conchita García-Patos. Quiero hacer mención especial a María del Carmen Sánchez y Arancha Llama-Palacios, ya que sin ellas, sin su dedicación, paciencia y esfuerzo no lo habría conseguido. Recordaré siempre las charlas en el laboratorio, las PCRs , las mil dudas que me surgían y que me aclaraban incansablemente una y otra vez. Encontré en ellas a dos amigas con las que estaré siempre en deuda.

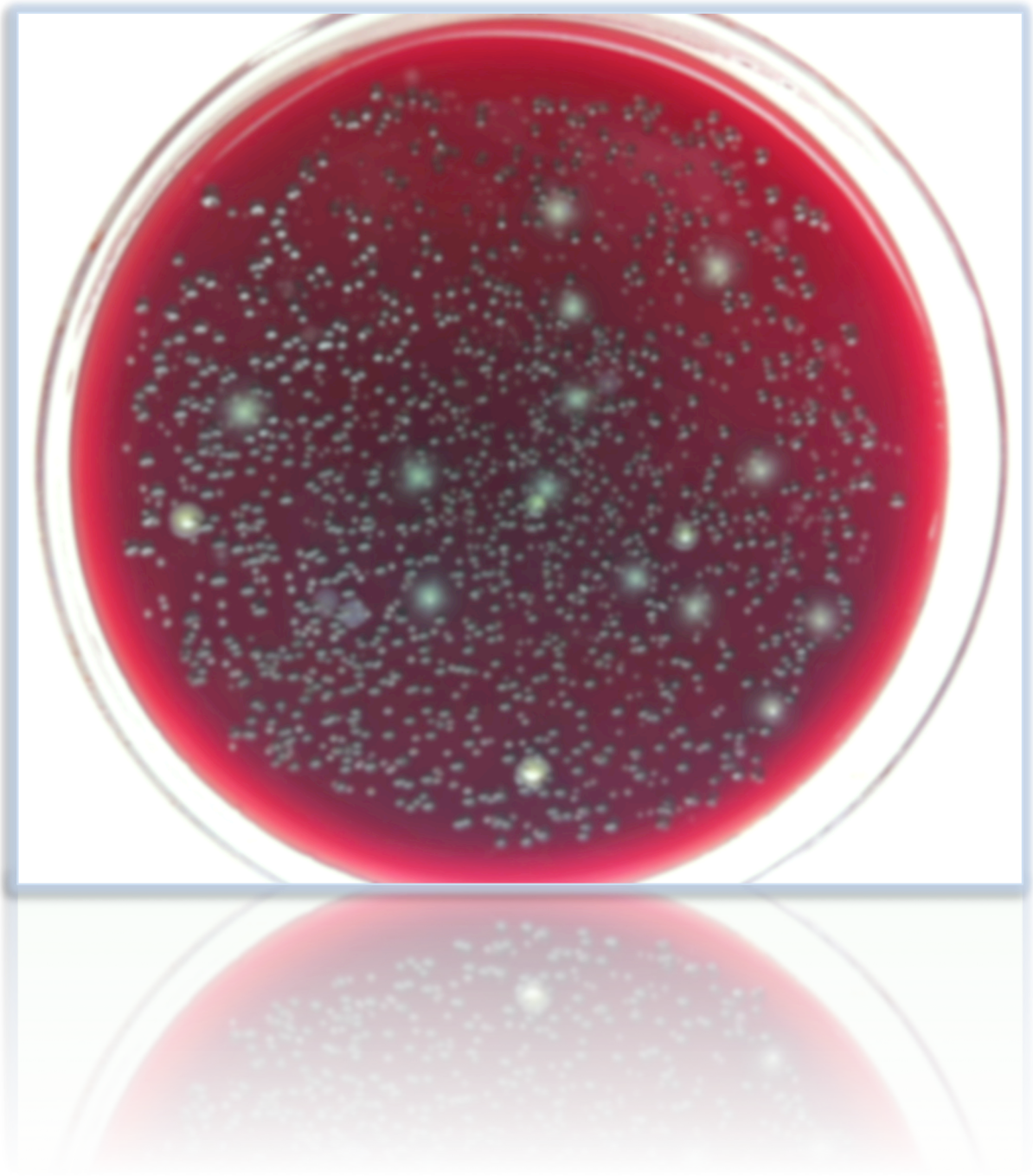
Mis padres, Andrés y Tana, siempre dicen que no les tengo que dar las gracias, pero quiero hacerlo; gracias por haber hecho posible que llegue hasta aquí, por animarme siempre a seguir adelante. Gracias a mis hermanos, Mar y Andrés, porque un rato de risas lo cura todo.

Por último y no menos importante, gracias a mi marido Nacho y a mi peque Bárbara. Gracias por entender todos los momentos sacrificados para poder llegar hasta aquí. Gracias a vuestro apoyo lo hemos conseguido. Esta tesis es de los tres.

Muchísimas gracias a todos.

Índice

Abreviaturas



ADN: ácido desoxirribonucleico.

CHT: cloramina T.

CHX: clorhexidina.

CLSM: microscopía láser confocal.

CFU: unidades formadoras de colonia.

CPC: cloruro de cetilpiridinio.

EEOO: aceites esenciales

LTSEM: microscopía electrónica de barrido a baja temperatura.

NaF: fluoruro de sodio.

NC: control negativo.

PBS: tampón fosfato salino.

PCR: reacción en cadena de la polimerasa.

PMA: monoazida de propidio.

PVM/MA: copolímero polovinil metil éter/ácido maleíco.

qPCR: reacción en cadena de la polimerasa en modalidad cuantitativa.

RCT: ensayo controlado aleatorizado.

SnF: fluoruro de estaño.

SHMP: hexametafosfato de sodio.

SLA: Sand-blasted, Large grit, Acid-etched.

TC: triclosán.

Summary

Background and Objectives

Biofilms are complex microbial communities developed on solid surfaces exposed to a wet environment. Oral biofilms are considered the etiologic agents of the most important oral diseases. In the oral cavity, different biofilms may be encountered attached to different types of surfaces including teeth, prosthetic devices and dental implants. The formation and maturation of biofilms over dental implant surfaces may have pathogenic implications in the development of peri-implant diseases, such as peri-implant mucositis or peri-implantitis.

In spite of the similarities between the biofilms on tooth or implant surfaces, some biofilm features may be attributed to the implant´s specific micro- and macroscopic surface characteristics. Previous *in vitro* and *in vivo* studies have reported that some surface characteristics like roughness, surface free energy, wettability and degree of sterilization may affect biofilm formation and the bacterial three- dimensional distribution, although there is controversy on the existence of the specific mechanism.

Similarly, if the surface can affect the formation and development of the biofilm, the effectiveness of different antiseptics over that biofilm can be also affected. With the purpose of studying the implant surface bacterial interactions, several *in vitro* biofilm models have been tested and validated. These systems have usually included one or two bacterial species and/or short-term evaluations (24 h or less), thus lacking the ability to study the dynamics of the biofilm maturation and its potential pathogenicity.

Our research group has developed and tested an *in vitro* model for complex biofilm formation on tooth (hydroxyapatite) surfaces, both under static and dynamic conditions, using six bacterial species comprising initial (*Streptococcus oralis* and

Actinomyces naeslundii), early (*Veillonella parvula*), secondary (*Fusobacterium nucleatum*) and late colonizers (*Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans)*.

The purpose of these series of studies were, first, to evaluate this *in vitro* biofilm model on implant surfaces, testing both titanium and zirconium surfaces, and to compare the obtained biofilms with the standard on hydroxyapatite surfaces. Using this biofilm model, our research group has recently tested the use of combined molecular techniques to study the antimicrobial effects of different substances, when applied to oral biofilms, in order to overcome some of the limitations of culture-based techniques. This method combines bacterial quantification through real-time polymerase chain reaction (qPCR) and the dye propidium monoazide (PMA), which discriminates between live and dead bacteria. The use of molecular techniques offers clear advantages: PCR-based techniques may enhance specificity and sensitivity over traditional culture-based techniques, as well as their ability to obtain results faster; however, a major disadvantage of PCR is the detection of DNA from both viable and dead bacterial cells, due to the relatively long persistence of DNA after cell death. To avoid this disadvantage, the qPCR technique was combined with PMA, which only can penetrate into bacterial cells with compromised membrane integrity, resulting in the capability to distinguish between viable and damaged cells by PCR.

The second objective was to compare the antibacterial effects of toothpastes, by developing a new methodology, which used the slurry method for applying the toothpaste on a multispecies subgingival *in vitro* oral biofilm model and the evaluation of its efficacy by qPCR and PMA.

Finally, as third objective, the number of viable bacteria of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and *Fusobacterium nucleatum* associated to a complex biofilm model formed on SLA titanium and zirconium oxide surfaces was compared, when exposed to different antiseptic agents [alcohol- free essential oils (EEOOs), cetylpyridinium chloride (CPC) and chlorhexidine (CHX) combined with CPC].

Results, Discussion and Conclusions

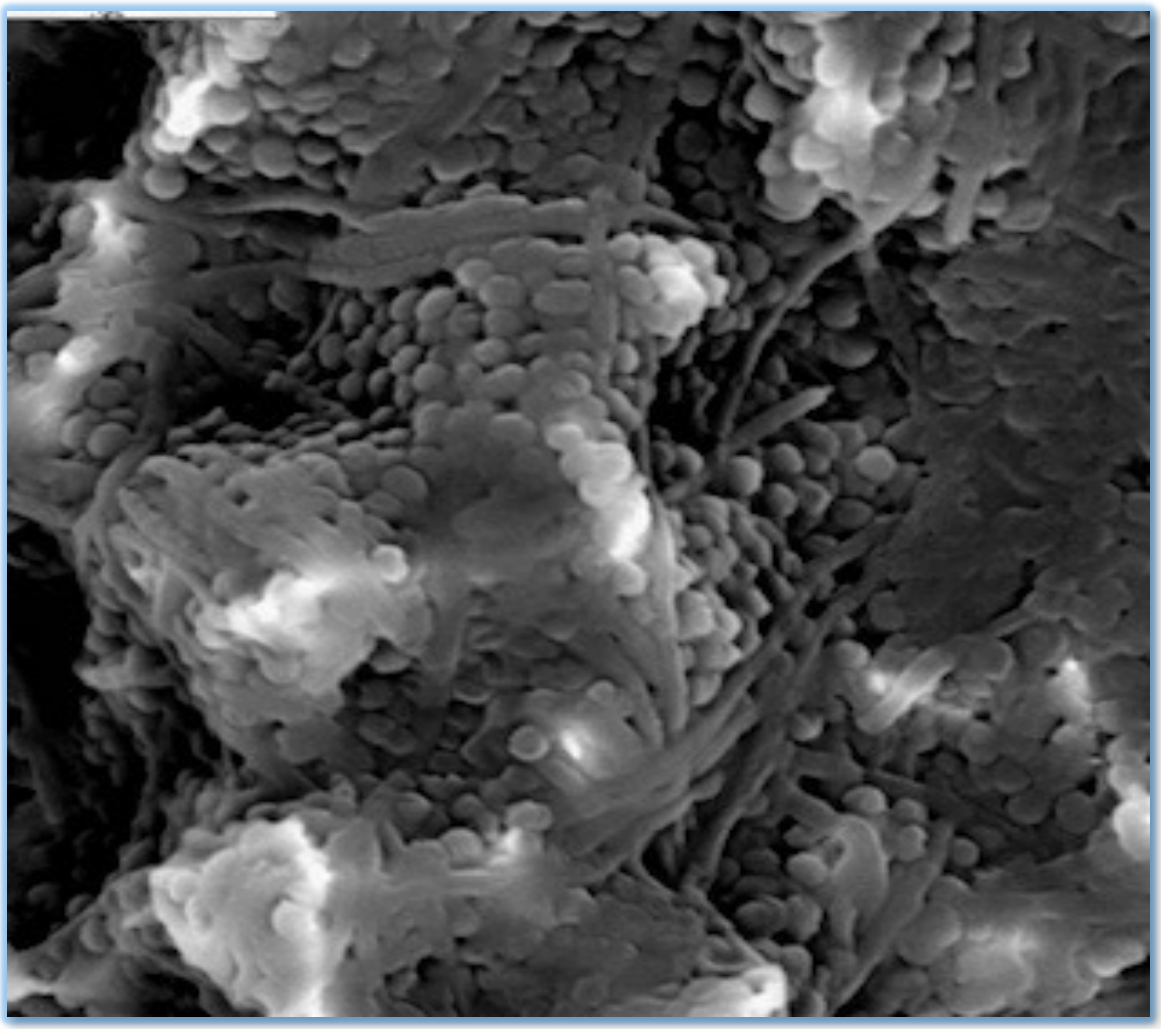
In the first investigation, the structure and bacterial kinetics of biofilms, developed *in vitro* on three different surfaces, were compared: hydroxyapatite, titanium and zirconium. The structural analysis showed that biofilms were developed on the three tested materials. Different surfaces, however, demonstrated differences in the biofilm tri-dimensional structure. This investigation has demonstrated that the formation and dynamics of an *in vitro* biofilm model was similar, irrespective of the surface of inoculation (hydroxyapatite, titanium or zirconium). There were significant differences, however, between the biofilms on hydroxyapatite, on one side, and those on titanium and zirconium surfaces, on the other side, in respect to the three dimensional organization of the biofilms and in the number of bacteria within the biofilms. This investigation has also shown that the use of confocal laser scanning microscopy (CLSM), low-temperature scanning electron microscopy (LTSEM) and qPCR allows the study of *in vitro* models, both in terms of structure and morphology, as well as bacterial dynamics and kinetics.

The difficulties to evaluate the effectiveness of toothpastes, in the second study, without the variability associated to tooth brushing, led to the proposal to prepare slurries of dentifrices, to use them as a rinse, avoiding the need to brush. This

technique has been applied previously in different studies, under non-brushing conditions or in other *in vitro* and *ex vivo* studies, which tested the different toothpastes converted into slurries, using them as mouth rinses and under non-brushing conditions. The present study confirmed the validity of the slurry method to compare toothpastes in an *in vitro* biofilm model. In addition, the validity of culture-independent molecular methods to assess the antimicrobial effects of antiplaque agents was demonstrated, since a PCR-based method was able to accurately detect and quantify viable bacteria after the antimicrobial treatment. The proposed model was able to detect significant differences in the antimicrobial effects of the tested toothpastes. The antimicrobial effects of toothpastes, in the format of slurries, can be compared in an *in vitro* multispecies biofilm model, using culture-independent microbiological techniques.

The results from the third investigation have shown that, even within the limitations of this in vitro study, *A. actinomycetencomitans, P. gingivalis* and *F. nucleatum* respectively suffer a similar decrease in their viability (viable colony forming units, CFU/mL) when are included in an *in vitro* multi-species biofilm formed on either Sand-blasted, Large grit, Acid-etched (SLA) titanium and zirconium surfaces, despite the significant differences in regards to the biofilm architecture, when were topically exposed to antimicrobial agents (CHX/CPC, EEOOs and CPC), whether the application was purely chemical or combined with agitation.

Resumen



Antecedentes y objetivos

Las biopelículas son complejas comunidades microbianas que se desarrollan en superficies sólidas en un medio húmedo . Las biopelículas orales están consideradas como los agentes etiológicos de las enfermedades orales más importantes. En la cavidad oral, se pueden hallar distintas biopelículas ligadas a diferentes tipos de superficies, incluyendo dientes, prótesis e implantes dentales. La formación y maduración de las biopelículas o biofilms sobre la superficie de implantes dentales puede tener implicaciones en el desarrollo de enfermedades periimplantarias como mucositis o periimplantitis.

A pesar de las semejanzas entre el biofilm sobre superficies dentarias o superficies de implantes, algunas características del biofilm podrían verse alteradas por el diseño macro y microscópico de los implantes. Estudios previos *in vitro* e *in vivo* han señalado que algunos parámetros como la rugosidad de la superficie, la energía libre superficial, la humectabilidad y el modo de esterilización de los implantes, son factores que afectan significativamente a la formación de la biopelícula y a la distribución tridimensional dentro de la misma. De igual manera, si la superficie puede afectar a la formación y desarrollo de la biopelícula, la efectividad de los diferentes antisépticos sobre esa biopelícula también puede verse afectada. Con el propósito de estudiar las interacciones bacterianas en la superficie de los implantes, se han probado y validado numerosos modelos de biofilm *in vitro*. Estos sistemas habitualmente incluyen una o dos especies bacterianas, o realizan evaluaciones en un período corto de tiempo (24 horas o menos), careciendo así de la capacidad para estudiar la dinámica de maduración del biofilm y su potencial patogenicidad. Nuestro Grupo de Investigación,

ha desarrollado y validado un modelo *in vitro* para la formación de un biofilm complejo sobre superficies dentarias (hidroxiapatita), bajo condiciones estáticas y dinámicas, usando seis especies bacterianas, que comprenden colonizadores iniciales (*Streptococcus oralis* y *Actinomyces naeslundii*), tempranos (*Veillonella parvula*), secundarios (*Fusobacterium nucleatum*) y tardíos (*Porphyromonas gingivalis* y *Aggregatibacter actinomycetencomitans*).

El propósito de esta serie de estudios fue, en primer lugar, evaluar el modelo de biofilm *in vitro* sobre superficies de implantes de titanio y zirconio y compararlo con el mismo biofilm formado sobre hidroxiapatita. Empleando este modelo de biofilm, nuestro grupo de investigación ha probado recientemente el uso de técnicas moleculares para el estudio del efecto antimicrobiano de distintas sustancias al aplicarlas a biofilms orales, con el fin de superar algunas de las limitaciones de las técnicas de cultivo. Este método combina la reacción en cadena de la polimerasa (PCR) cuantitativa a tiempo real (qPCR), con la tinción discriminante de monoazida de propidio (PMA), lo que permite distinguir entre bacterias vivas y muertas. El uso de estas técnicas moleculares basadas en la PCR pueden mejorar la especificidad y la sensibilidad sobre las técnicas de cultivo clásicas, así como su habilidad para obtener resultados más rápidamente. Sin embargo, una desventaja importante de la PCR es que detecta ADN de células vivas y muertas. Para evitarlo, la técnica de qPCR se puede combinar con PMA, el cual sólo puede penetrar en bacterias muertas con compromiso de la integridad de su membrana, siendo capaz de distinguir así entre células vivas y muertas con qPCR.

El segundo objetivo ha sido comparar el efecto antibacteriano de diferentes dentífricos, desarrollando una nueva metodología, que consiste en emplear el método

de “slurry” para aplicar el dentífrico en el modelo de biofilm subgingival *in vitro* y evaluar su eficacia a través de qPCR y PMA.

Por último, como tercer objetivo, se ha comparado el número de bacterias vivas de *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* y *Fusobacterium nucleatum* asociadas a un modelo de biofilm formado sobre superficies SLA de titanio y óxido de zirconio, tras ser expuestas a diferentes agentes antisépticos [aceites esenciales sin alcohol (EEOOs), cloruro de cetilpiridinio (CPC) y clorhexidina (CHX) combinada con CPC].

Resultados, Discusión y Conclusiones

En la primera investigación, se compararon la estructura y cinética bacteriana de los biofilms, desarrollados *in vitro* sobre tres superficies diferentes: hidroxiapatita, titanio y zirconio. El análisis estructural mostró que los biofilms de desarrollaron en los tres materiales testados. Sin embargo se encontraron diferencias en la estructura tridimensional de los biofilms en las distintas superficies. Esta investigación ha demostrado que la formación y dinámica de un modelo de biofilm *in vitro* fue similar, sin tener en cuenta la superficie de inoculación (hidroxiapatita, titanio o zirconio). Sin embargo, se han encontrado diferencias significativas entre los biofilms de hidroxiapatita, por un lado, y los formados sobre superficies de titanio y zirconio por otro lado, en lo que se refiere a la organización tridimensional de los biofilms y el número de bacterias. Esta investigación también ha mostrado que el uso de microscopía láser confocal (CLSM), microscopía electrónica de barrido a baja temperatura (LTSEM) y qPCR permite el estudio de modelos *in vitro*, tanto en términos de estructura y morfología, así como en dinámica y cinética bacteriana.

Las dificultades para evaluar la efectividad de los dentífricos sin la variabilidad asociada al cepillado condujo al propósito de prepararlos en forma de “slurry”, para emplearlos como un enjuague, evitando la necesidad de cepillar. Esta técnica se ha aplicado previamente en diferentes estudios, bajo condiciones de no cepillado**,** o en estudios *in vitro* y *ex vivo* que testaron las diferentes pastas en forma de “slurry”, empleándolos como enjuagues y bajo condiciones de no cepillado. El presente estudio confirma que el método de “slurry” es válido para comparar dentífricos en un modelo de biofilm *in vitro*. Además, está demostrada la validez de la técnica de qPCR para medir los efectos antimicrobianos de los agentes antiplaca, ya que fue capaz de detectar y cuantificar exactamente las bacterias viables después del tratamiento antimicrobiano.

Los resultados de la tercera investigación han mostrado que, a pesar de las limitaciones de emplear un modelo *in vitro*, las especies bacterianas *Aggregatibacter actinomycetencomitans*, *Porphyromonas gingivalis* y *Fusobacterium nucleatum*, incluidas en el modelo de biofilm *in vitro* formado sobre superficies de titanio (SLA) y óxido de zirconio, sufrieron un descenso similar en su vitalidad al ser expuestas a los diferentes agentes antimicrobianos, ya fuera en aplicación directa o con agitación.

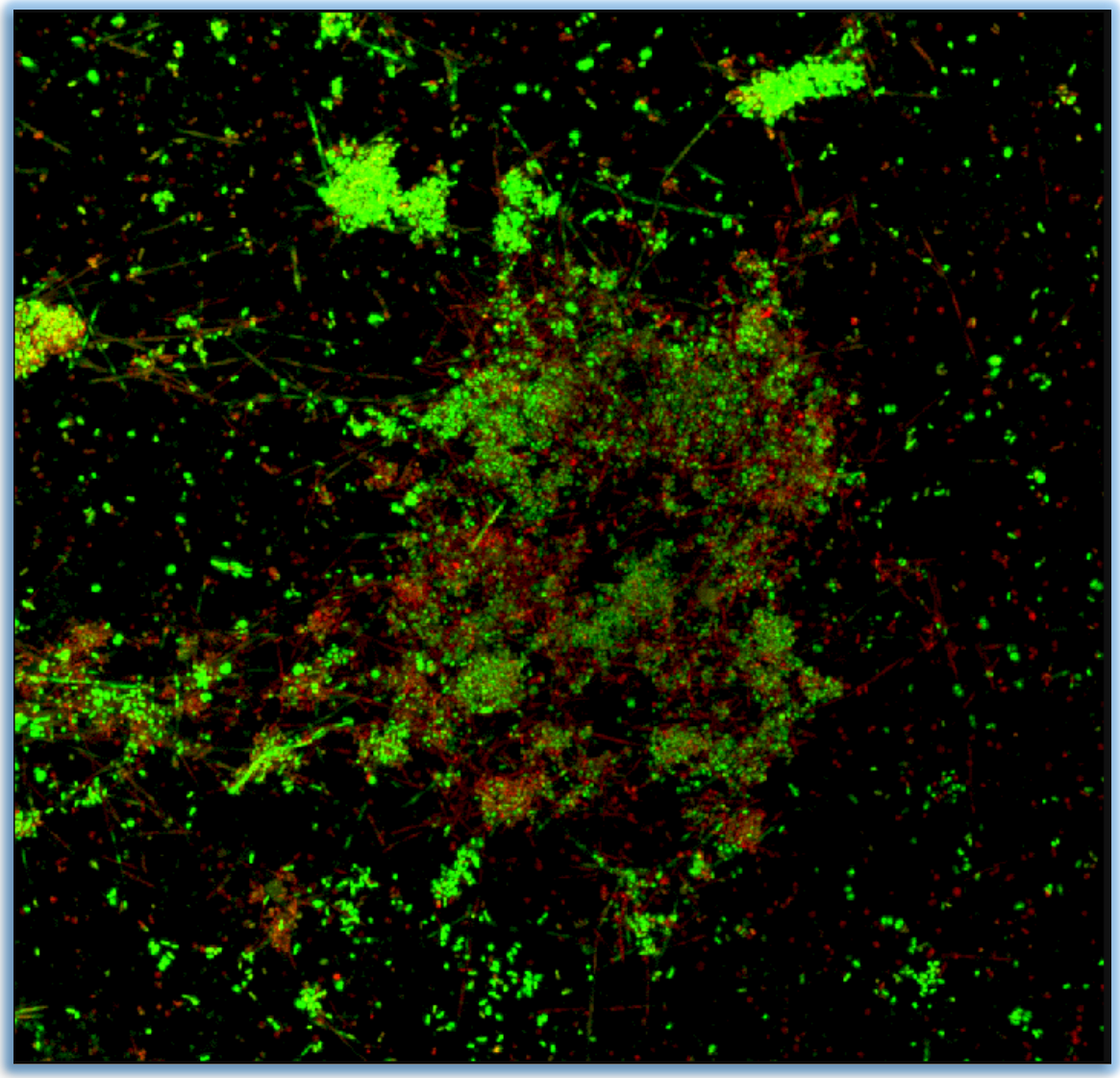
La presente tesis doctoral se basa en las siguientes investigaciones originales publicadas en revistas científicas:

Artículo original 1. M.C. Sánchez, A. Llama-Palacios, **E. Fernández,** E. Figuero, M.J. Marín, R. León, V. Blanc, D. Herrera, M. Sanz. An *in vitro* biofilm model associated to dental implants: structural and quantitative analysis of *in vitro* biofilm formation on different dental implant surfaces. *Dental Materials* (2014);30(10):1161-71. doi: 10.1016/j.dental.2014.07.008.

Artículo original 2. E. Fernández, MC. Sánchez, A. Llama-Palacios , M. Sanz, D. Herrera. Antibacterial effects of toothpastes evaluated in an *in vitro* biofilm model. *Oral Health and Preventive Dentistry* (2016). Aceptado para publicación.

Artículo original 3. MC. Sánchez, **E. Fernández,** A. Llama-Palacios, E. Figuero, V. Blanc, R. León, D. Herrera, M. Sanz. Response to antiseptic agents of periodontal pathogens in in vitro biofilms on titanium and zirconium surfaces. *Dental Materials* (2017) Feb 22. pii: S0109-5641(16)30523-1. doi: 10.1016/j.dental.2017.01.013. [Epub ahead of print].

Introducción



1 Los conceptos de placa bacteriana y de biopelículas bacterianas

Desde la primera observación a través del microscopio, por parte de A. van Leeuwenhoek en 1676, se ha ido conociendo la complejidad del ecosistema que forma la placa bacteriana (Porter 1976) y numerosos estudios han evaluado la composición de la placa empleando el microscopio óptico y electrónico, técnicas de cultivo y, más recientemente, técnicas moleculares basadas en el ADN. Todas estas técnicas reforzaban las observaciones iniciales de van Leeuwenhoek, en las que la placa bacteriana estaba compuesta por una mezcla compleja de especies bacterianas (Socransky et al. 1998).

Ya en los inicios del siglo XX, A.T. Henrici y, más tarde, J.W. Costerton, apuntaron la existencia de diferentes poblaciones de microorganismos viviendo sobre distintas superficies (Lebeaux et al. 2013), y su relación con la salud bucodental. Los investigadores en el ámbito dental, durante los últimos 120 años, han intentado comprender la naturaleza microbiana de las enfermedades orales. Su forma de entender la placa bacteriana y los microorganismos que la constituyen ha variado desde una hipótesis de placa específica a una hipótesis de placa no específica, y de nuevo a una teoría de patógenos periodontales específicos en la placa. Estos cambios en la forma de comprender la placa bacteriana afectan directamente en las estrategias empleadas para prevenir y tratar las enfermedades periodontales (Overman 2000).

A partir de la segunda mitad del siglo XX, la comunidad científica en el ámbito dental centró el foco de atención en la placa bacteriana, poniendo especial interés en los factores contribuyentes a la diversidad de ecosistemas, incluyendo pH, potencial de óxido-reducción y factores nutricionales (Thomas y Nakaishi 2006). A partir de 1990, con la llegada del microscopio láser confocal y el microscopio electrónico de barrido,

se consiguió un mayor conocimiento de la placa dental y su estructura (Marsh y Bradshaw 1995; Marsh y Bradshaw 1997; Costerton et al. 1987; Costerton et al. 1993; Costerton et al. 1994; Donlan 2002; Donlan y Costerton 2002; Bernimoulin 2003; Serrano-Granger y Herrera 2005).

Hoy en día sabemos que las bacterias en la boca se asocian formando "biofilms" o biopelículas, constituyendo éstas el estado más habitual de las bacterias en la mayoría de los ecosistemas naturales (Stoodley et al. 2002). Una definición ampliamente aceptada de una biopelícula bacteriana es la postulada por Donlan y Costerton (2002), que la describen como «una comunidad bacteriana inmersa en un medio líquido, caracterizada por bacterias que se hallan unidas a un substrato o superficie, o unas a otras, que se encuentran embebidas en una matriz extracelular producida por ellas mismas, y que muestran un fenotipo alterado en cuanto al grado de multiplicación celular o la expresión de sus genes».

Las enfermedades iniciadas por biopelículas no son exclusivas de la cavidad oral, pero sí son, probablemente, las enfermedades infecciosas más comunes que afectan a la mayor parte de la población mundial, entre las que destacan las enfermedades periodontales (gingivitis y periodontitis) y la caries dental (Socransky y Haffajee 2002; Sheiham y Netuveli 2002; Singh et al. 2010). En ambas patologías, los microorganismos se organizan como un consorcio de multi-especies, clásicamente llamado "placa dental", el cual tiene profundas implicaciones en su etiología (Guggenheim et al. 2001). Hoy en día, se pueden encontrar en la cavidad oral más de 750 especies bacterianas distintas y, entre ellas, hay grupos específicos que son los responsables del inicio y/o progresión de las enfermedades periodontales, incluyendo gingivitis y periodontitis (Singh et al. 2010).

2 Formación de la biopelícula en dientes e implantes dentales

La asociación de las bacterias en las biopelículas orales no es aleatoria sino que hay asociaciones específicas entre especies bacterianas. Socransky y colaboradores examinaron más de 13.000 muestras de placa subgingival procedente de 185 adultos e identificaron seis grupos de especies bacterianas asociadas, que incluían colonizadores iniciales, como *Streptococcus oralis* (con capacidad para fijarse a diferentes receptores de la película dental y proveer de receptores a especies como *Fusobacterium nucleatum*), intermedios y tardíos (Socransky et al. 1998) (Figura 1).

Fig. 1 Esquema de la asociación entre especies subgingivales tomado de Socransky et al. 1998. Los datos provienen de 13.261 muestras de placa subgingival tomadas de 185 adultos. Los complejos de la izquierda están compuestos por especies que se cree que colonizan la superficie dentaria y proliferan en estadios tempranos. El complejo naranja se cree que es el puente entre los colonizadores iniciales y las especies del complejo rojo que dominan en etapas tardías (tomado de Socransky et al. 1998).

La biopelícula oral, por lo tanto, se desarrolla por un proceso de colonización selectiva, reproducible y secuencial (Aas et al. 2005; Díaz et al.2006; Kolenbrander et al. 1990). En los colonizadores iniciales predominan especies de *Actinomyces*, *Neisseria, Prevotella, Streptococcus* y *Veillonella* (Díaz et al. 2006; Nyvad y Kilian 1987). Los colonizadores secundarios, como las fusobacterias, co-agregan con los colonizadores iniciales y hacen de puente para la co-agregación de nuevas bacterias, destacando (por su potencial patógeno) las especies *Aggregatibacter actinomycetemcomitans,*

Porphyromonas gingivalis y *Tannerella forsythia* (Kolenbrander y London 1993; Kolenbrander et al. 2002). Sin embargo, la mayor parte de una biopelícula la constituye la matriz de exopolisacáridos, que es producida por las propias bacterias que forman parte de la biopelícula y que representa de un 50 a un 95% del peso seco (Sutherland 1999). Todas las biopelículas contienen exopolisacáridos, aunque una característica distintiva de las biopelículas orales es que muchos de los microorganismos pueden sintetizarlos y degradarlos (Socransky y Haffajee 2002).

Las biopelículas orales pueden considerarse entre las más complejas de las que existen en la naturaleza. Esto se debe, en gran medida, a la superficie no cambiante/descamable del diente, que permite una colonización persistente y así da la oportunidad de desarrollar ecosistemas complejos; además, la abundancia de nutrientes disponibles en la cavidad oral y la gran capacidad de las especies orales para co-agregarse entre sí pueden explicar esta complejidad (Socransky y Haffajee 2002).

Las bacterias que se encuentran en la saliva se podrían considerar como bacterias planctónicas (aunque diversos autores consideran a la saliva como el fluido que baña diferentes ecosistemas orales), sin embargo, las bacterias que se encuentran sobre superficies duras como dientes, prótesis, restauraciones e implantes, forman una película gelatinosa que se ha denominado clásicamente placa dental (Bernimoulin 2003). La placa dental es un ejemplo de biopelícula formada por combinaciones de cientos de especies bacterianas que compiten para colonizar las superficies de la cavidad oral (Sedlacek y Walker 2007). Las biopelículas son el medio de crecimiento preferido de muchas de estas especies bacterianas, ya que proporcionan una serie de ventajas: la principal es la protección que ofrece la biopelícula a la especie colonizadora frente a factores ambientales, mecanismos de defensa del huésped y

sustancias potencialmente tóxicas como productos químicos letales o antibióticos. Las biopelículas pueden facilitar también el procesado de nutrientes, la alimentación cruzada y el desarrollo de un ambiente físico-químico apropiado (Socransky y Haffajee 2002). Por ello, la capacidad de unirse entre sí y adherirse a superficies es una estrategia fundamental de supervivencia para la mayoría de los organismos procariotas (Marsh 2005).

Por lo tanto, las biopelículas son complejas comunidades microbianas que se desarrollan en superficies sólidas que se encuentran en un medio húmedo (Belibasakis et al. 2015) y la cavidad oral cumple estos requisitos, pudiendo encontrarse diferentes biopelículas unidas a distintas superficies como dientes, prótesis o implantes dentales (Lee y Wang 2010; Busscher et al. 2010; Lang y Berglundh 2011; Belibasakis et al. 2015).

Los implantes dentales, introducidos en las últimas décadas como soporte para prótesis fijas o removibles, con la finalidad de reponer dientes perdidos (Berglundh et al. 2002), no están libres de problemas a medio y largo plazo, especialmente los relacionados con la acumulación de biofilms bacterianos en sus superficies. Cualquier material extraño que es implantado es susceptible de ser colonizado por microorganismos (Costerton et al. 2005), así que es posible la colonización bacteriana en cualquier lugar expuesto del implante, incluso en su superficie rugosa expuesta indeseablemente en la cavidad oral (Serino y Ström 2009; Ioannidis et al. 2015). La formación de biofilm alrededor de los implantes parece ser crítico para el desarrollo de enfermedades periimplantarias y podría ser responsable de alterar la biocompatibilidad de la superficie de los implantes (Renvert et al. 2008). Por ello, a pesar de que hoy en día el reemplazo de dientes por implantes dentales se realiza con

éxito, con un elevado porcentaje de supervivencia del implante (Violant et al. 2014; Lang et al. 2011), se describen frecuentemente complicaciones técnicas y biológicas (Quirynen et al. 2002; Roos-Jänsaker et al. 2006; Jung et al. 2008).

La formación y maduración de la biopelícula en la superficie de los implantes se ha relacionado con la etiología de las enfermedades periimplantarias, de igual manera que la biopelícula subgingival se asocia a gingivitis y periodontitis (Quirynen et al. 2002; Lee y Wang 2010; Busscher et al. 2010; Lang y Berglundh 2011; Belibasakis et al. 2015; Subramani et al. 2009). Las enfermedades periimplantarias se han definido como un proceso inflamatorio que puede ser, fundamentalmente, de dos tipos: mucositis periimplantaria, en la que existe una lesión inflamatoria que se limita a la mucosa circundante sin pérdida ósea, con presencia de sangrado al sondaje y profundidad de sondaje igual o superior a 4 mm (Renvert et al. 2007) y periimplantitis, en la que además de los hallazgos previos, se produce pérdida progresiva de hueso de soporte (Ferreira et al. 2006; Karoussis et al. 2004).

La biopelícula en la superficie de los implantes es similar a la que se forma sobre superficies dentarias; sin embargo, algunas características de la misma pueden verse alteradas por el diseño macro y microscópico de los implantes (Lang et al. 2011; Quirynen et al. 2005; Ioannidis et al. 2014; Sánchez et al. 2014). Estudios *in vivo* (Rimondini et al. 1997; Scarano et al. 2003; Keller et al. 1998) e *in vitro* (Sánchez et al. 2014; Wu-Yuan et al. 1995; Drake et al. 1999; Grössner- Schreiber et al. 2001; Mabboux et al. 2004) sobre la influencia de las características de la superficie de los implantes dentales en la formación de la biopelícula, han mostrado que parámetros como la rugosidad de la superficie, la energía libre superficial, la humectabilidad y el modo de esterilización de los implantes, son factores que afectan significativamente a

la formación de la biopelícula y a la distribución tridimensional dentro de la misma, mostrando diferencias, entre otras, en su espesor, formación de la matriz de polisacáridos extracelular así como en la organización de las células bacterianas (Sánchez et al. 2014). Estudios recientes que evalúan biopelículas sobre pilares de implantes, con superficies diferentes en cuanto a composición y rugosidad, muestran que existe correlación entre la rugosidad de la superficie y la biomasa viable en la biopelícula (Hahnel et al. 2014, 2015).

Estos resultados coinciden con otros modelos *in vitro,* donde la rugosidad de la superficie influye significativamente en la formación de la biopelícula (Quirynen et al. 1994; Schmidlin et al. 2013; Zhao et al. 2014; Albouy et al. 2012), mientras que, en otros estudios, en los que se compararon diferentes superficies (de titanio y zirconio, con menor atención a la rugosidad, la energía libre superficial aparentó ser el factor más importante que determina la adhesión bacteriana inicial (Mabboux et al. 2004; Al-Radha et al. 2012). De la misma manera, en algunos modelos, a medida que la rugosidad de la superficie aumenta, se produce un aumento en la colonización bacteriana (Whitehead et al. 2005; Teughels et al. 2006; Xing et al. 2015), mientras que en otros estudios, cierta topografía del titanio puede reducir la adhesión bacteriana al mismo tiempo que promueve la óseo-integración (Puckett et al. 2010; Gordon et al. 2015). Otro factor a tener en cuenta es que la colonización bacteriana temprana puede verse influida también por la pureza del titanio y no sólo por la topografía de su superficie (Violant et al. 2014).

3 El control de las biopelículas bacterianas

Mantener una boca libre de enfermedad es, hoy en día, más importante que nunca a tenor de los estudios publicados en los últimos años, en los que se relacionan la salud oral y sistémica (Chapple y Genco 2013; Sanz et al. 2013). La inflamación asociada con periodontitis puede contribuir a problemas a nivel sistémico tan relevantes como las enfermedades cardiovasculares y diabetes, entre otras (Singh et al. 2010; Gurav 2016). Para la mayoría de las personas, el método más efectivo, seguro y económico de control de los biofilms orales, es el cepillado dental suplementado con un dentífrico. El control de la placa supragingival comienza con su eliminación mecánica a través del cepillado. Sin embargo, el control de placa únicamente con el cepillado dental es una meta difícil para la mayoría de los pacientes (Van Strydonck et al. 2006). La Asociación Dental Americana define el mantenimiento de una buena higiene oral como el cepillado de dientes durante dos minutos, dos veces al día, sumado al uso de la seda dental una vez al día (Ayad et al. 2010).

Si el mantenimiento de la salud bucodental y periodontal (en prevención primaria y secundaria) tiene recomendaciones establecidas, como se ha expuesto previamente, aparentemente el manejo preventivo a través de diferentes estrategias, como la higiene oral diaria, serían también necesarias para prevenir las enfermedades periimplantarias. Sin embargo, si los biofilms periimplantarios fueran diferentes a los formados alrededor de los dientes, estas diferencias podrían tener un impacto en la susceptibilidad de las biopelículas al control mecánico o a los agentes antimicrobianos, lo cual podría afectar al éxito de las medidas preventivas comunes (por ejemplo, el uso de antisépticos como parte de la higiene oral) y también a los enfoques terapéuticos (incluyendo el uso de antimicrobianos locales o sistémicos). Además, las superficies

más propensas a albergar biopelículas complejas pueden ser más susceptibles para el desarrollo de enfermedades periimplantarias (Serino y Ström 2009; Ioannidis et al. 2014).

Sin embargo, incluso en relación con los dientes, se ha demostrado que sólo con el cepillado y la seda no siempre es suficiente para mantener una boca sana. Por esta razón, se empezaron a añadir agentes quimioterapéuticos en las formulaciones de los dentífricos/colutorios para aumentar la eficacia antiplaca y/o antigingivitis (Ayad et al. 2010).

El dentífrico es el vehículo ideal para transportar sustancias antibacterianas, pues se va a usar de manera conjunta con el cepillado mecánico. Por su parte, los colutorios suelen ser más fáciles de formular (en cuanto a la biodisponibilidad de los agentes activos) y tienen muy buena aceptación por parte de la población (Serrano et al. 2015). Estos productos quimioterapéuticos deben ser seguros, efectivos en la reducción de placa y gingivitis, deben tener sustantividad, deberían afectar sólo a la flora patógena y tener un sabor agradable (Hancock y Newell 2001). Los fluoruros han demostrado ser eficaces en la prevención de la caries, mientras que agentes antiplaca como la clorhexidina, cloruro de cetilpiridinio, aceites esenciales, fluoruro de estaño y triclosán, entre otros, han demostrado actividad antiplaca y antigingivitis (Arweiler et al. 2002; Serrano et al. 2015). Estos productos, sin embargo, deben de acreditar su efectividad en estudios bien diseñados, que comienzan con la evaluación de la actividad antimicrobiana *in vitro*. La mayoría de los estudios, en este sentido, se realizan sobre bacterias en forma planctónica, cuando, según se ha explicado, las bacterias diana están organizadas en forma de biofilms, con lo que se hace imprescindible el desarrollo de modelos reproducibles para evaluar *in vitro* la actividad antibacteriana de

diferentes formulaciones (dentífricos o colutorios), frente a bacterias organizadas en biofilms.

Los estudios *in vitro* constituyen una importante herramienta para evaluar agentes antibacterianos y pueden proveer interesantes ideas sobre su potencial eficacia clínica. Agentes con actividad antibacteriana demostrable *in vitro* pueden ser efectivos sobre los mismos microorganismos *in vivo*, pero agentes sin actividad antibacteriana demostrable *in vitro* es improbable que la muestren *in vivo* (Ciancio y Panagakos 2010).

Con el propósito de estudiar las interacciones bacterianas en la superficie de los implantes, se han probado y validado numerosos modelos de biofilm *in vitro* (Burgees et al. 2010; Lee et al. 2011; Al- Radha et al. 2012; Rimondini et al. 2002; Schmidlin et al. 2013), pero estos sistemas habitualmente incluyen solamente una o dos especies bacterianas, o realizan evaluaciones en un período corto de tiempo (24 horas o menos), careciendo así de la capacidad para estudiar la dinámica de maduración del biofilm y su potencial patogenicidad. El Grupo de Investigación ETEP ("Etiología y Terapéutica de las Enfermedades Periodontales"), de la Universidad Complutense de Madrid, ha desarrollado y probado un modelo *in vitro* para la formación de un biofilm complejo sobre superficies dentarias (hidroxiapatita), bajo condiciones estáticas y dinámicas, usando seis especies bacterianas, que comprenden colonizadores iniciales (*Streptococcus oralis* y *Actinomyces naeslundii*), tempranos (*Veillonella parvula*), secundarios (*Fusobacterium nucleatum*) y tardíos (*Porphyromonas gingivalis* y *Aggregatibacter actinomycetencomitans*) (Sánchez et al. 2011; Blanc et al. 2014).

Este modelo de biofilm multi-especies es un modelo reproducible, técnicamente sencillo de preparar, mantener y analizar. Es un modelo válido para estudiar no sólo el desarrollo, estructura y dinámica del biofilm subgingival sino también para realizar estudios comparativos de diferentes productos antimicrobianos (Sánchez et al. 2011). Esto es especialmente relevante, teniendo en cuenta que las bacterias organizadas en biofilms son más resistentes frente agentes antimicrobianos que en forma planctónica (Sedlacek y Walker 2007; Costerton et al. 1987; Socransky y Haffajee 2002; Verkaik et al. 2011). De hecho, la mayoría de los estudios *in vitro* con dentífricos se han realizado sobre especies bacterianas aisladas, mientras que hay una falta de estudios *in vitro* que empleen modelos de biofilm subgingival incluyendo los principales patógenos relacionados con el desarrollo de la periodontitis.

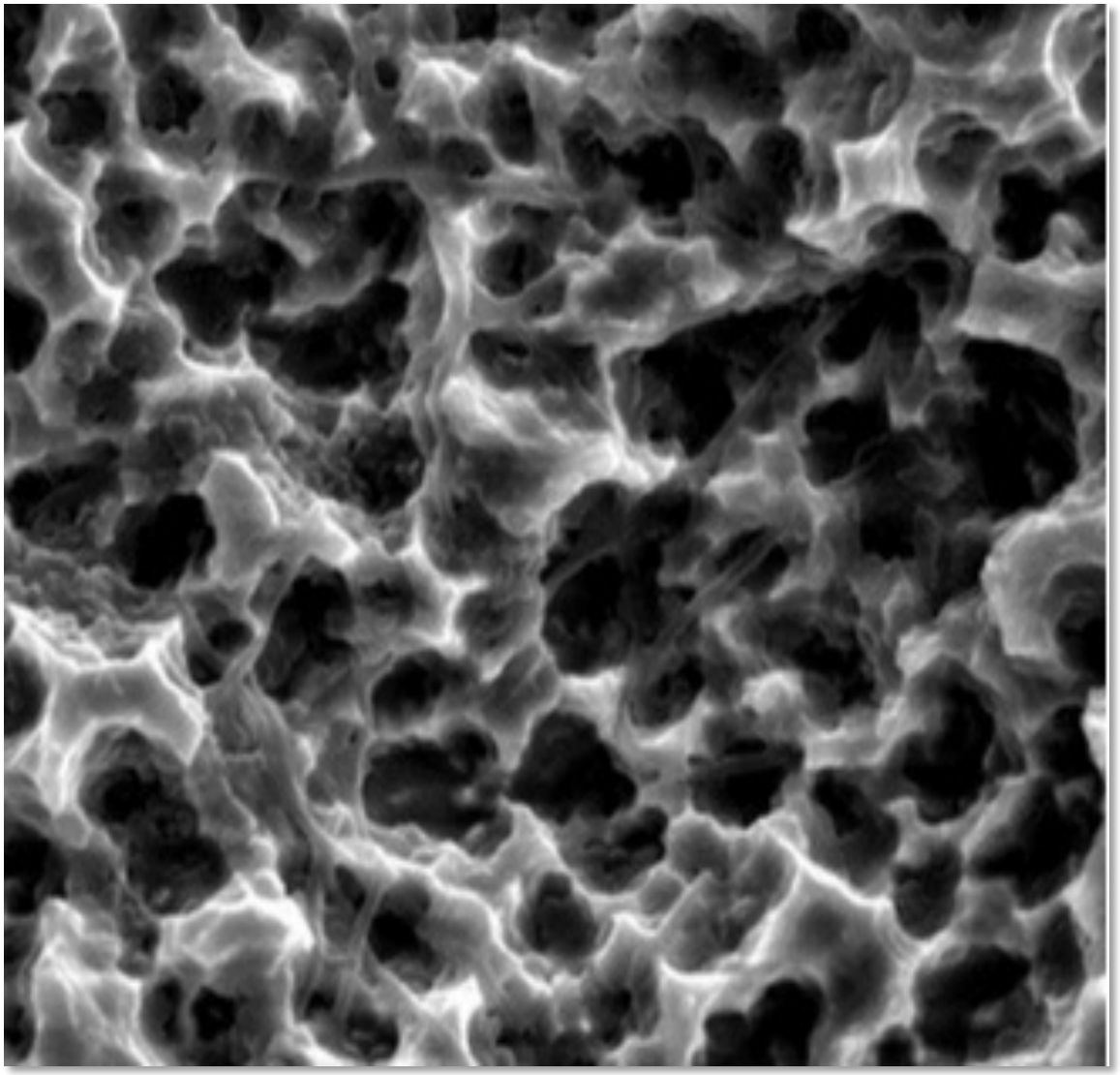
Para superar las dificultades inherentes al estudio *in vitro* con dentífricos, se ha propuesto el método denominado comúnmente como "slurry". Los dentífricos se preparan como "slurries" y se aplican *in vitro* al biofilm maduro subgingival. En otros estudios, los dentífricos en forma de "slurry" han sido aplicados a un biofilm oral de 16 horas (Verkaik et al. 2011), y también se han usado en modelos *in vivo,* como el modelo de "re-crecimiento de placa" (Addy et al. 1997; Binney et al. 1997; Moran et al. 2005; Van Strydonck et al. 2006; He et al. 2010), o en estudios *in vitro* y/o *ex vivo* que miden la concentración mínima inhibitoria de diferentes dentífricos sobre especies bacterianas asociadas con caries, periodontitis y halitosis (Haraszthy et al. 2010; Emani et al.2014). Sin embargo es difícil encontrar estudios *in vitro* que actúen sobre un modelo de biofilm subgingival que incluya bacterias involucradas en el desarrollo de la periodontitis.

Muchos de los estudios mencionados emplearon técnicas de cultivo para evaluar el efecto antimicrobiano de los dentífricos, con las limitaciones derivadas de éstas técnicas, como son, por ejemplo, los largos tiempos de incubación de algunas bacterias, la necesidad de medios de cultivo específicos para determinados grupos bacterianos o la habilidad necesaria para identificar morfológicamente las colonias (Breeuwer y Abee 2000). Para superar algunas de las limitaciones del cultivo, se pueden usar las técnicas moleculares, como la reacción en cadena de la polimerasa (PCR) cuantitativa a tiempo real (qPCR). Si esta técnica se combina, además, con la tinción discriminante de monoazida de propidio (PMA), permite distinguir entre bacterias vivas y muertas, lo que es especialmente relevante para estudios sobre el efecto de antimicrobianos. Estos métodos han sido probados recientemente para estudiar el efecto antimicrobiano aplicado a biofilms orales (Sánchez et al. 2014). La razón fundamental es que estas técnicas moleculares basadas en la PCR pueden mejorar la especificidad y la sensibilidad sobre las técnicas de cultivo clásicas, así como su habilidad para obtener resultados más rápidamente (Olsen et al. 1995). Sin embargo, una desventaja importante de la PCR es que detecta ADN de células vivas y muertas, debido a la relativamente larga persistencia del ADN después de la muerte celular (Nogva et al. 2003; Rudi et al. 2005; Nocker et al. 2006; Cawthorn y Witthuhn 2008). Para evitar esta desventaja, la técnica de qPCR se puede combinar con PMA, el cual sólo puede penetrar en bacterias muertas con compromiso de la integridad de su membrana, siendo capaz de distinguir así entre células vivas y muertas con qPCR (Nocker et al. 2006; Cawthorn y Witthuhn 2008; Nocker et al. 2009).

Respecto a los modelos de evaluación *in vitro* en superficies de implantes dentales, la evidencia científica disponible es muy limitada. Aunque las formulaciones con agentes

antimicrobianos específicos como clorhexidina, aceites esenciales o cloruro de cetil-piridinio, de manera coadyuvante con la eliminación mecánica de la placa, han mostrado beneficios clínicos en la prevención de las enfermedades periimplantarias y en el tratamiento de la mucositis periimplantaria (Graziani et al. 2012), todavía existe controversia sobre si la topografía de la superficie del implante y su tratamiento químico pueden influir en la capacidad de estos agentes antimicrobianos para actuar sobre la composición del biofilm y su patogenicidad.

Justificación, Hipótesis y Objetivos



1. Justificación

Las enfermedades bucodentales, en general, y las periodontales, en particular, son especialmente relevantes por su alta prevalencia y por las consecuencias que generan a nivel bucodental y por sus posibles consecuencias sistémicas.

El inicio y la progresión de las enfermedades bucodentales más importantes (especialmente las enfermedades periodontales) están asociados con bacterias de las biopelículas orales, en concreto con la placa bacteriana. Esto mismo ocurre con las enfermedades periimplantarias, que afectan a los implantes dentales y pueden causar la destrucción del tejido óseo que los sostiene, y la eventual pérdida del implante y de la prótesis que sostiene.

Las estrategias preventivas y terapéuticas en las enfermedades periodontales se basan en el control de las biopelículas orales y, aunque el control mecánico es el tratamiento de referencia, en algunos pacientes y patologías no es suficiente, lo que ha conducido a la introducción del uso de productos antimicrobianos. Para usarlos de manera coadyuvante a la higiene mecánica (cepillado), se pueden formular en forma de dentífricos o colutorios.

Dado que la biopelícula que se forma alrededor de los implantes dentales puede ser diferente a la que se forma sobre los dientes, es necesario desarrollar estrategias preventivas y terapéuticas específicas, y están deben de ser validadas en estudios adecuadamente diseñados. La evaluación, por ejemplo, de dentífricos y colutorios, comienza con estudios de análisis de la capacidad antimicrobiana *in vitro,* que deberían hacerse sobre modelos de biofilm validados sobre superficies de implantes.

El Grupo de Investigación ETEP (“Etiología y Terapéutica de las Enfermedades Periodontales”), de la Universidad Complutense de Madrid, ha desarrollado un modelo

de biofilm *in vitro* usando seis especies bacterianas (Sánchez et al. 2011), que comprenden colonizadores iniciales (*Streptococcus oralis* y *Actinomyces naeslundii*), colonizadores tempranos (*Veillonella parvula*), secundarios (*Fusobacterium nucleatum*) y tardíos (*Porphyromonas gingivalis* y *Aggregatibacter actinomycetencomitans*), demostrando un patrón de colonización bacteriana y maduración similar al desarrollo *in vivo* de la biopelícula subgingival.

Para poder analizar la capacidad antimicrobiana *in vitro* , es fundamental emplear métodos de cuantificación selectiva de células vivas, combinando la técnica de la reacción en cadena de la polimerasa cuantitativa (qPCR) y el compuesto monoazida de propidio (PMA) (Sánchez et al. 2014).

2. Hipótesis

Hipótesis general

El modelo de biofilm *in vitro* desarrollado y probado por el Grupo de Investigación ETEP de la Universidad Complutense de Madrid (Sánchez et al. 2011; Blanc et al. 2014), permitirá estudiar las diferencias en las biopelículas formadas sobre distintas superficies, así como el efecto de distintos antisépticos sobre el biofilm formado tanto en superficies de implantes, como en superficies de dientes (hidroxiapatita), y se comprobará la idoneidad del mismo empleado como una herramienta para desarrollar biofilms periimplantarios *in vitro*.

Hipótesis específicas

Las biopelículas que se forman sobre superficies de titanio y zirconio son diferentes a los que se forman sobre superficies de dientes (hidroxiapatita) (Estudio #1).

Los efectos antimicrobianos de los dentífricos, en forma de "slurry", pueden ser comparados en el modelo de biofilm *in vitro*, empleando técnicas de biología molecular (Estudio # 2).

El efecto de diferentes agentes antisépticos formulados en colutorios (aceites esenciales sin alcohol, clorhexidina, cloruro de cetilpiridinio), sobre el número de bacterias viables de *Aggregatibacter actinomycetencomitans*, *Porphyromonas gingivalis* y *Fusobacterium nucleatum* mostrará diferencias en función, no solo del agente empleado, sino también de la superficie sobre la que se forma el biofilm (Estudio #3).

3. Objetivos

El objetivo general de este trabajo fue evaluar un modelo reproducible de biofilm *in*

vitro, ya desarrollado, sobre superficies de implantes dentales e hidroxiapatita, y validarlo en diferentes estudios comparativos.

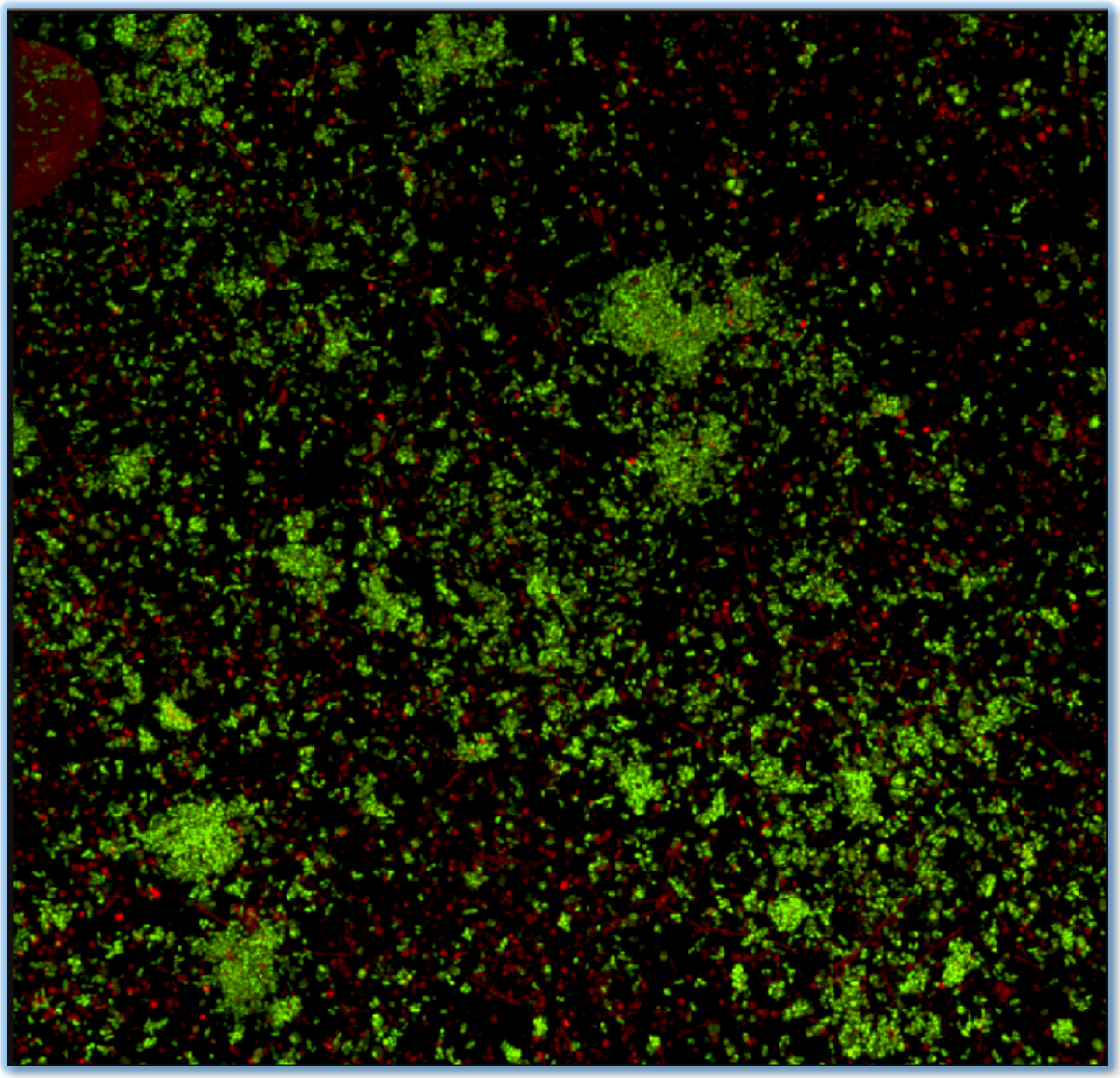
De manera específica, se establecieron los siguientes objetivos:

1. Comparar la estructura y cinética bacteriana en un modelo de biofilm *in vitro* desarrollado en tres superficies diferentes: hidroxiapatita, titanio y zirconio (Estudio #1).

2. Comparar el efecto antibacteriano de diferentes dentífricos, en forma de "slurry", en un modelo de biofilm *in vitro* (Estudio #2).

3. Evaluar el impacto en la susceptibilidad del modelo de biofilm *in vitro*, formado sobre distintas superficies de implantes dentales, frente a diferentes antimicrobianos en forma de colutorio (Estudio #3).

Materiales y Métodos



Los Materiales y Métodos, al igual que los resultados de las investigaciones realizadas en la presente tesis, han sido publicados como artículos científicos originales en tres publicaciones independientes en diferentes revistas científicas*:*

Estudio #1. M.C. Sánchez, A. Llama-Palacios, E. Fernández, E. Figuero, M.J. Marín, R. León, V. Blanc, D. Herrera, M. Sanz. An *in vitro* biofilm model associated to dental implants: Structural and quantitative analysis of *in vitro* biofilm formation on different dental implant surfaces. *Dental Materials* (2014);30(10):1161-71. doi: 10.1016/j.dental.2014.07.008.

Estudio #2. E. Fernández , MC. Sánchez , A. Llama-Palacios , M. Sanz and D. Herrera. Antibacterial effects of toothpastes evaluated in an *in vitro* biofilm model. *Oral Health and Preventive Dentistry* (2016). (aceptado para publicación).

Estudio #3. MC. Sánchez, E. Fernández, A. Llama-Palacios, E. Figuero, V. Blanc, R. León, D. Herrera and M. Sanz. Response to antiseptic agents of periodontal pathogens in in vitro biofilms on titanium and zirconium surfaces. *Dental Materials* (2017) Feb 22. pii: S0109-5641(16)30523-1. doi: 10.1016/j.dental.2017.01.013. [Epub ahead of print].

1 Estudio 1

Sánchez MC, Llama-Palacios A, Fernández E, Figuero E, Marín MJ, León R, Blanc V, Herrera D, Sanz M. An *in vitro* biofilm model associated to dental implants: Structural and quantitative analysis of *in vitro* biofilm formation on different dental implant surfaces. *Dental Materials* (2014);30(10):1161-71. doi: 10.1016/j.dental.2014.07.008.

RESUMEN

Antecedentes y objetivo: El impacto de las distintas superficies de los implantes en el desarrollo del biofilm oral es, todavía hoy, desconocido. El objetivo de esta investigación ha sido evaluar *in vitro* el desarrollo de un modelo de biofilm sobre superficies de implantes de titanio y zirconio y compararlo con el mismo biofilm formado sobre superficie de hidroxiapatita.

Material y Método: Se emplearon seis bacterias de referencia para desarrollar una biopelícula *in vitro* sobre discos estériles de titanio, zirconio e hidroxiapatita, cubiertos con saliva, en placas multipocillo de cultivo celular. Las bacterias seleccionadas representan colonizadores primarios (*S. oralis* y *A. naeslundii*), tempranos (*V. Parvula*), secundarios (*F. nucleatum*) y tardíos (*P. gingivalis* y *A. actinomycetemcomitans*). El desarrollo de las biopelículas (tiempo de crecimiento de 1 a 120 horas), se estudió a través de una técnica vital de fluorescencia combinada con microscopía láser confocal (CLSM, por sus siglas en inglés). Como variables se estudiaron el espesor de la biopelícula, el porcentaje de vitalidad celular y el número de bacterias empleando el análisis de la varianza.

Resultados: Las bacterias se adhirieron y maduraron en las tres superficies de una manera similar. Sin embargo, las tres superficies demostraron diferencias tanto en el

espesor, la disposición de la matriz de polisacáridos extracelular así como en la organización de las células bacterianas.

Conclusión: Aunque la formación y la dinámica de crecimiento de la biopelícula *in vitro* ha sido similar en las tres superficies (hidroxiapatita, titanio y zirconio), ha habido diferencias estadísticamente significativas con respecto al espesor y la estructura tridimensional.

DENTAL MATERIALS XXX (2014) XXX–XXX

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An *in vitro* biofilm model associated to dental implants: Structural and quantitative analysis of *in vitro* biofilm formation on different dental implant surfaces

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ARTICLE INFO

Article history:
Received 7 January 2014
Received in revised form
26 March 2014
Accepted 15 July 2014
Available online xxx

Keywords:
Peri-implant biofilm
Zirconium
Titanium
Hydroxyapatite
LTSEM
CLSM
qPCR

ABSTRACT

Objectives. The impact of implant surfaces in dental biofilm development is presently unknown. The aim of this investigation was to assess *in vitro* the development of a complex biofilm model on titanium and zirconium implant surfaces, and to compare it with the same biofilm formed on hydroxyapatite surface.

Methods. Six standard reference strains were used to develop an *in vitro* biofilm over sterile titanium, zirconium and hydroxyapatite discs, coated with saliva within the wells of pre-sterilized polystyrene tissue culture plates. The selected species used represent initial (*Streptococcus oralis* and *Actinomyces naeslundii*), early (*Veillonella parvula*), secondary (*Fusobacterium nucleatum*) and late colonizers (*Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*). The developed biofilms (growth time 1 to 120 h) were studied with confocal laser scanning microscopy using a vital fluorescence technique and with low-temperature scanning electron microscopy. The number (colony forming units/biofilm) and kinetics of the bacteria within the biofilm were studied with quantitative PCR (qPCR). As outcome variables, the biofilm thickness, the percentage of cell vitality and the number of bacteria were compared using the analysis of variance.

Results. The bacteria adhered and matured within the biofilm over the three surfaces with similar dynamics. Different surfaces, however, demonstrated differences both in the thickness, deposition of the extracellular polysaccharide matrix as well as in the organization of the bacterial cells.

Significance. While the formation and dynamics of an *in vitro* biofilm model was similar irrespective of the surface of inoculation (hydroxyapatite, titanium or zirconium), there were significant differences in regards to the biofilm thickness and three-dimensional structure.

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http://dx.doi.org/10.1016/j.dental.2014.07.008
0109-5641/

1. Introduction

Biofilms are complex microbial communities developed on solid non-shed surfaces exposed to a wet environment [1]. In the oral cavity bacteria may attach to different types of surfaces including teeth, prosthetic devices and dental implants. Biofilms on dental implants have not been characterized, although similar to the etiological association between dental biofilms and the most prevalent oral infections [2–4], the formation and maturation of biofilms on dental implant surfaces may have pathogenic implications in the development of peri-implant diseases, such as peri-implant mucositis or peri-implantitis [2,3].

Studies on the microbiota around dental implants have shown that when bacteria colonize the peri-implant crevice soon after implant placement, the dominant species are streptococci and members of the yellow and purple complexes, such as *Actinomyces* spp., soon developing a polymicrobial community [5,6]. Similarly, the microorganisms associated to the implant surface in presence of healthy peri-implant tissues are predominantly Gram-positive cocci and rods [2]. As the biofilm matures, members of the red, orange, and green complexes colonize [2,5–9] and in clinical situations associated with failing implants or peri-implant diseases, there is a net predominance of orange and red complex species [6,10–12].

Even though changes in the composition of the peri-implant microbiota have been associated with the pathogenesis of peri-implant diseases [13], there is controversy on how the implant surface characteristics may affect bacterial colonization and biofilm formation, thus potentially influencing the initiation and progression of peri-implant diseases [14,15].

With the purpose of studying the implant surface bacterial interactions, several *in vitro* biofilm models have been tested and validated [16–20]. These systems have usually included one or two bacterial species or short-term evaluations (24 h or less), thus lacking the ability to study the dynamics of the biofilm maturation and its potential pathogenicity. Our research has developed and tested an *in vitro* model for complex biofilm formation on tooth (hydroxyapatite) surfaces, both under static and dynamic conditions, using six bacterial species comprising (*Streptococcus oralis* and *Actinomyces naeslundii*), early (*Veillonella parvula*), secondary (*Fusobacterium nucleatum*) and late colonizers (*Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*) [21,22]. We, therefore, aim in this investigation to test this *in vitro* biofilm model on implant surfaces, testing both titanium and zirconium surfaces, and to compare the obtained biofilms with the standard on hydroxyapatite surfaces.

2. Material and methods

2.1. Bacterial strains and culture conditions

Standard reference strains of *S. oralis* CECT 907T, *V. parvula* NCTC 11810, *A. naeslundii* ATCC 19039, *F. nucleatum* DMSZ 20482, *A. actinomycetemcomitans* DSMZ 8324 and *P. gingivalis* ATCC 33277 were used. Bacteria were grown on blood agar plates (Blood Agar Oxoid No 2; Oxoid, Basingstoke, UK), supplemented with 5% (v/v) sterile horse blood (Oxoid), 5.0 mg mL^{-1} hemin (Sigma, St. Louis, MO, USA) and 1.0 mg mL^{-1} menadione (Merck, Darmstadt, Germany) in anaerobic conditions (10% H_2, 10% CO_2, and balance N_2) at 37 °C for 24–72 h.

2.2. Material specimens

Three different surface materials were used: sterile calcium hydroxyapatite discs with a 7 mm of diameter and a thickness 1.8 mm (standard deviation, SD = 0.2) (Clarkson Chromatography Products, Williamsport, PA, USA); sterile titanium discs, grade 2, SLA (Sand-blasted, Large grit, Acid-etched) surface of 5 mm of diameter (Straumann; Institut Straumann AG, Basel, Switzerland); sterile zirconium oxide (ZrO_2) discs, surface of 5 mm of diameter with a rough micro surface obtained after chemical treatment with a hot solution of hydrofluoric acid according to a proprietary process of Institut Straumann AG (Institut Straumann AG, Basel, Switzerland). The resultant rough surface topography has a S_a value of 0.55 mm (SD = 0.01). This rough surface topography when evaluated with SEM has similar topography as Ti-SLA surface implants, although zirconia surfaces seemed to have a flatter profile with less porosity [S_a value of Ti-SLA surface of 1.17 mm (SD = 0.04)] [23].

2.3. Saliva preparation

Un-stimulated saliva was obtained from healthy volunteers in 10 mL aliquots at least 1.5 h after eating, drinking or tooth brushing. Each saliva sample was treated with 2.5 mmol L^{-1} DL-dithiothreitol (Sigma) for 10 min with continuous stirring in order to reduce salivary protein aggregation. It was then centrifuged (10 min, 4 °C, 9000 × *g*) and the obtained supernatant were diluted (1:1) with phosphate buffered saline (PBS). The sample was then filtered and sterilized through a 0.22 μm pore size Millex GV low-protein-binding filter X50 (Millipore, Millipore Corporation Bedford, USA) and stored at -20 °C. The efficacy of this protocol was assessed by plating processed saliva samples onto supplemented blood agar plates for 72 h at 37 °C and confirmed by the lack of any bacterial growth on either aerobically or anaerobically incubated plates.

2.4. Biofilm development

Biofilms were grown on the three surfaces as previously described by Sánchez et al. [21]. In brief, pure cultures of each bacteria were grown anaerobically in a protein-rich medium containing brain-heart infusion (BHI) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) supplemented with 2.5 g L^{-1} mucin (Oxoid), 1.0 g L^{-1} yeast extract (Oxoid), 0.1 g L^{-1} cysteine (Sigma), 2.0 g L^{-1} sodium bicarbonate (Merck), 5.0 mg mL^{-1} hemin (Sigma), 1.0 mg mL^{-1} menadione (Merck) and 0.25% (v/v) glutamic acid (Sigma). The bacterial growth was harvested at mid-exponential phase (measured by spectrophotometry), and a mixed bacteria suspension in modified BHI medium containing 10^3 colony forming units (CFU) mL^{-1} for *S. oralis*, 10^5 CFU mL^{-1} for *V. parvula* and *A. naeslundii*, and 10^6 CFU mL^{-1} for *F. nucleatum*, *A. actinomycetemcomitans* and *P. gingivalis* was prepared. Sterile

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discs were coated with treated saliva for 4 h at 37 °C in sterile plastic tubes, and then placed in the wells of a 24-well tissue culture plate (Greiner Bio-one, Frickenhausen, Germany). Each well was inoculated with 1.5 mL mixed bacteria suspension prepared and incubated in anaerobic conditions (10% H_2, 10% CO_2, and balance N_2) at 37 °C for 1, 12, 24, 48, 72, 96 and 120 h. Plates containing only culture medium were also incubated to check for sterility.

2.5. Analysis of biofilms by confocal laser scanning microscopy (CLSM)

Three independent trials (on three different occasions) with trios of biofilms were carried out. Before CLSM analysis, the discs were sequentially rinsed in 2 mL of sterile PBS (immersion time per rinse, 10 s) three times, in order to remove non-adherent bacteria. Non-invasive confocal imaging of fully hydrated biofilms was carried out using a fixed-stage TCS SP2 confocal microscope (Leica Microsystems, Milton Keynes, United Kingdom) incorporating a 488 nm Ar/Ar–Kr laser scan head mounted on a vibration-free platform. The objective lenses used were 63× water-immersion lenses (Leica Microsystems). Specimens were stained with LIVE/DEAD® BacLightTM Bacterial Viability Kit solution (Molecular Probes B. V., Leiden, The Netherlands) at room temperature. The 1:1 fluorocrome ratio with a staining time of 9 ± 1 min was used to obtain the optimum fluorescence signal at the corresponding wave lengths (Syto9: 515–530 nm; propidium iodide (PI): >600 nm). At least three separate and representative locations on the discs covered with biofilm were selected for these measurements (based on the presence of stacks or "towers" identified in the confocal view field). Within each area, the thickest point was measured by determining the upper and lower limits of the biofilm. The CLSM software was set to take a z-series of scans (xyz) of 1 μm thickness (8 bits, 1024 × 1024 pixel). Image stacks were analyzed with the Leica Confocal Lite® software (Leica Microsystems). In order to quantify the biomass and cell viability within the biofilm, total fluorescent staining of the confocal micrographs was analyzed using a specific image analysis software program (MetaMorph® 7.6; Molecular Devices Corporation, Sunnyvale, CA, USA). Fluorescence intensity thresholds were manually set for each of the fluorescent colors.

2.6. Analysis of biofilms by low-temperature scanning electron microscopy (LTSEM)

Three independent trials (on three different occasions) with trios of biofilms were carried out. For this analysis, the discs were sequentially rinsed in 2 mL of sterile PBS (immersion time per rinse, 10 s) three times, in order to remove non-adherent bacteria. The specimens were then fixed onto the specimen holder of the cryo-transfer system using liquid nitrogen and then transferred to a preparation unit *via* an air-lock transfer. The frozen specimens were transferred directly *via* a second air lock to the microscope cold stage, where they were etched for 2 min by raising the temperature to −90 °C. After ice sublimation, the etched surfaces were sputter-coated with gold in the preparation unit and then transferred onto the cold stage of the scanning electron microscope chamber. Surfaces were observed at −135 °C with a DMS 960 scanning electron microscope (Digital Scanning Microscope, Zeiss).

2.7. DNA isolation and quantitative polymerase chain reaction (qPCR)

Before the DNA isolation, the discs were sequentially rinsed in 2 mL of sterile PBS (immersion time per rinse, 10 s) three times, in order to remove non-adherent bacteria. Biofilm DNA was isolated from all samples using a commercial kit (MolYsis Complete5; Molzym GmbH & CoKG, Bremen, Germany), following manufacturer's instructions (the protocol for bacterial DNA extraction was followed from step 6, avoiding preliminary steps). The hydrolysis probes 5′ nuclease assay PCR method was used for detecting and quantifying the bacterial DNA. Primers and probes were obtained by Life Technologies Invitrogen (Carlsbad, CA, USA), Applied Biosystems (Carlsbad, CA, USA) and Roche (Roche Diagnostic GmbH; Mannheim, Germany) and were targeted against 16S *rRNA* gene (Table 1). The qPCR amplification was performed in a total reaction mixture volume of 20 μL. The reaction mixtures contained 10 μL of 2× master mixture (LC 480 Probes Master; Roche), optimal concentrations of primers and probe (900, 900 and 300 nM for *S. oralis*; 300, 300 and 300 nM for *A. naeslundii*; 750, 750 and 400 nM for *V. parvula*; 300, 300 and 200 nM for *A. actinomycetemcomitans*; 300, 300 and 300 nM, for *P. gingivalis* and 600, 600 and 300 nM for *F. nucleatum*), and 5 μL of DNA from samples. The negative control was 5 μL of sterile water [no template control (NTC)] (Water PCR grade, Roche). The samples were subjected to an initial amplification cycle of 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min Analyses was performed with a LightCycler® 480 II thermocycler (Roche). The plates used in the study were FramStar 480 of natural frame and white wells (4titude; The North Barn; Damphurst Lane, UK), sealed by QPCR Adhesive Clear Seals (4titude).

Each DNA sample was analyzed in duplicate. Quantification cycle (*Cq*) values, previously known as cycle threshold (*Ct*) values, describing the PCR cycle number at which fluorescence rises above the baseline, were determined using the provided software package (LC 480 Software 1.5; Roche). Quantification of viable cells by qPCR was based on standard curves, following a protocol previously described [27]. The correlation between *Cq* values and CFU/mL was automatically generated through the software (LC 480 Software 1.5; Roche).

All assays were developed with a linear quantitative detection range established by the slope range of 3.3–3.6 cycles/log decade, $r^2 > 0.997$ and an efficiency range of 1.9–2.0.

Measures to avoid carryover DNA were established, such as establishing barrier methods, using flow cabin and physical separation of pre- and post-PCR procedures. In spite of this, when NTC was detectable, the limit of detection was established on the last value of the standard curve that holds five cycles of difference with NTC.

2.8. Statistical analyses

The selected outcome variables to compare biofilms formed on different surfaces were micrometers of height, percentage of vitality and CFU/biofilm. An experiment-level analysis was performed for each study parameter ($n = 9$ for CLSM results and

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Table 1 – Primers and probes used for quantification of genomic DNA from the target bacteria. Primers and probes were targeted against 16S rRNA gene.

Bacteria	Sequence (5′–3′)	Length (bp)	Reference
So			
Forward	CAACGATACATAGCCGACCTGAG	97	Present
Reverse	TCCATTGCCGAAGATTCC		study
Probe	6FAM-CTCCTACGGGAGGCAGCAGTAGGGA-BBQ		
Vp			
Forward	TGCTAATACCGCATACGATCTAACC	66	[24]
Reverse	GCTTATAAATAGAGGCCACCTTTCA		
Probe	6FAM-CTATCCTCGATGCCGA-TAMRA		
An			
Forward	GGCTGCGATACCGTGAGG	103	Present
Reverse	TCTGCGATTACTAGCGACTCC		study
Probe	6FAM-CCCTAAAAGCCGGTCTCAGTTCGGAT-BBQ		
Pg			
Forward	GCGCTCAACGTTCAGCC	67	[25]
Reverse	CACGAATTCCGCCTGC		
Probe	6FAM-CACTGAACTCAAGCCCGGCAGTTTCAA-TAMRA		
Aa			
Forward	GAACCTTAC CTACTCTTGACATCCGAA	80	[26]
Reverse	TGCAGCACCTGTCTCAAAGC		
Probe	6FAM-AGAACTCAGAGATGGGTTTGTGCCTTAGGG-TAMRA		
Fn			
Forward	GGATTTATTGGGCGTAAAGC	162	[26]
Reverse	GGCATTCCTACAAATATCTACGAA		
Probe	6FAM-CTCTACACTTGTAGTTCCG-TAMRA		

So, *S. oralis*; Vp, *V. parvula*; An, *A. naeslundii*; Aa, *A. actinomycetemcomitans*; Fn, *F. nucleatum*; Pg, *P. gingivalis*.

$n = 3$ for qPCR results). Shapiro–Wilk goodness-of-fit tests and distribution of data were used to assess normality. Data were expressed as means, SD and 95% confidence intervals (95% CI). To compare the effects of the material surface at different exposure times on micrometers of height, percentage of vitality and CFU/biofilm, one-way analysis of variance and *post hoc* testing with Bonferroni's correction for multiple comparisons were used.

Results were considered statistically significant at $p < 0.05$. A software package (IBM SPSS Statistics 19.0; IBM Corporation, Armonk, NY, USA) was used for all data analysis.

3. Results

3.1. *Biofilm structure and viability assessed by CLSM and LTSEM*

The morphogenesis of the multispecies biofilm formed on saliva-coated hydroxyapatite, titanium and zirconium discs was examined by CLSM and LTSEM at different times (1, 12, 24, 48, 72, 96 and 120 h). Its three-dimensional architecture was associated with the early bacterial accumulation and the subsequent build up in an extracellular polysaccharides (EPS)-rich matrix. Figs. 1–3 depicts the biofilm formation over each material, and Table 2 shows the corresponding percentages of viable bacteria and biofilm thickness reached at the different time intervals until a mature state was reached.

One hour after inoculation, individual cells and multicellular aggregates could be identified sparsely attached to the three tested surfaces. After 12 h, a denser bacterial population was deposited and formed discontinuous layers of bacteria adhered to the three studied surfaces (Fig. 1). Bacterial cells were arranged either as short streptococcal chains (Fig. 1, red arrows) or as multicellular aggregates. Spindle-shaped rods, suggestive of *F. nucleatum* could be recognized inside the biofilms (Fig. 1, white arrows). Table 2 depicts the thickness of the biofilms (in μm) and percentage of cell vitality after 12 h in each surface. Statistically significant differences were observed for the height of the biofilm at 12 h of development when the titanium was compared to hydroxyapatite and zirconium surfaces ($p < 0.05$, in both cases). No statistically significant differences were found when comparing the vitality of biofilms among the three materials ($p = 1.00$).

The observation with LTSEM revealed notable differences in the initial structure of the biofilms according to the material-surface. On hydroxyapatite discs, bacterial cells spread across the disk surface without an evident formation of EPS matrix: its structural organization was based primarily on bacterial cell-to-cell binding (co-aggregation) (Fig. 1D). In contrast, on the titanium surfaces, the biofilm was rich in EPS with discernible micro-colonies as buds, surrounded by filamentous bacteria (*F. nucleatum*) (Fig. 1E). The EPS is recognized as a thin film coating the bacterial cells forming a compact mass of greater brightness (Fig. 1E, blue arrow). Fig. 2A shows EPS matrix as a dense mass packing the bacterial cells in young titanium biofilms. Conversely, biofilm structure on zirconium surfaces was characterized by a formation of an EPS with a fibrillar aspect (Fig. 1F, blue arrow) connecting individual bacterial cells including short streptococcal chains and micro-colonies of filamentous bacteria (Fig. 1F, yellow arrow).

During the following 24 h of *in situ* biofilm formation, the complexity as well as the size of these bacterial communities increased. With LTSEM, the biofilms exhibited a structure over

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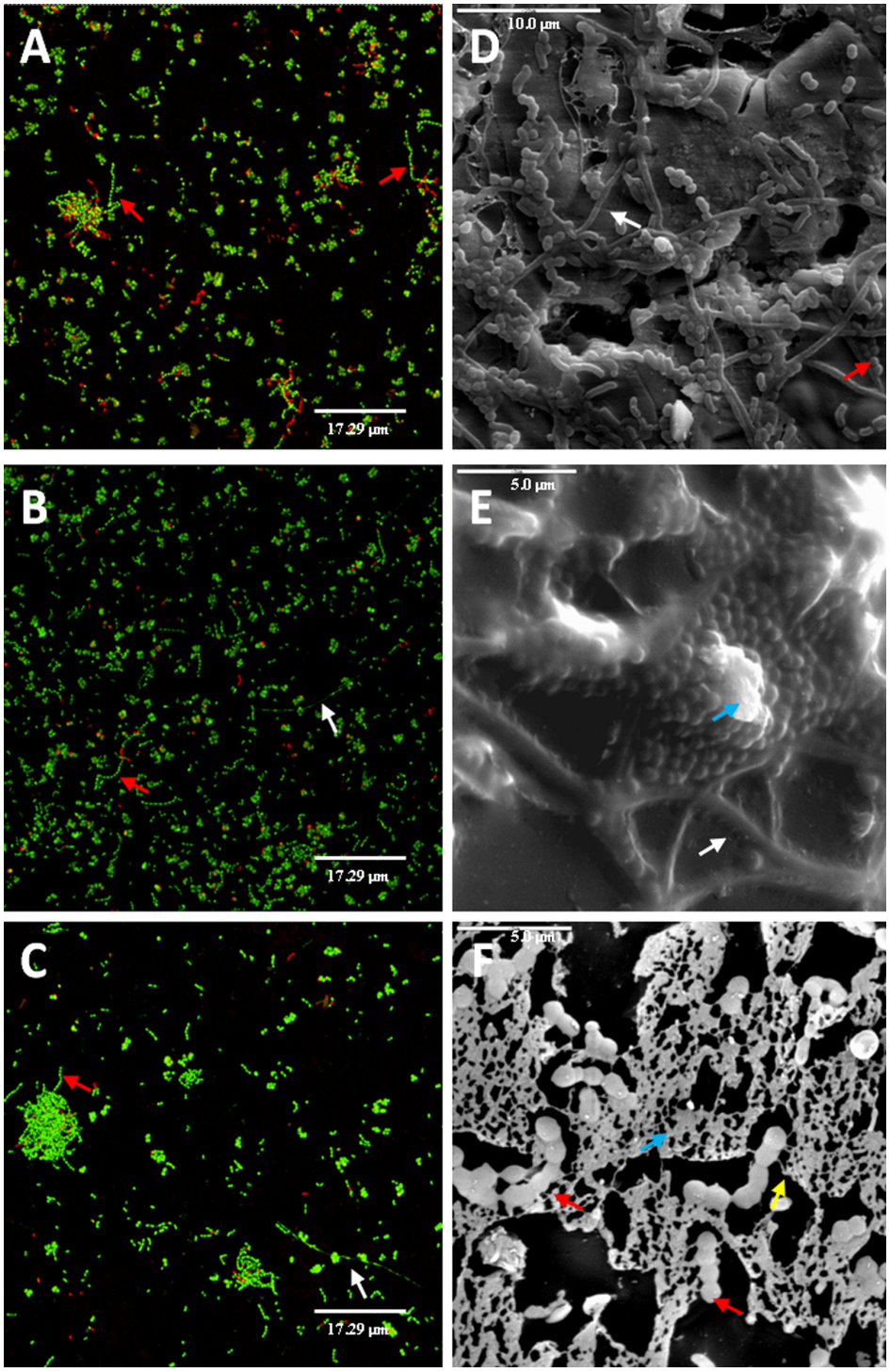

Fig. 1 – CLSM (A, B and C) and LTSEM (D, E and F) images of biofilms growth after 12 h of incubation, over hydroxyapatite (A and D), titanium (B and E) and zirconium (C and F) surfaces. Maximum projection (CLSM) as well as close up images (LTSEM) of biofilms show bacterial cells spread across the disk surface of the three surfaces mentioned above. Cells were arranged either as individual cells, as short streptococcal chains (red arrows) or as multicellular aggregates; also, spindle-shaped rods, indicating *F. nucleatum*, could be recognized inside the biofilms (white arrows). Blue arrow indicates the presence of EPS-matrix, recognized in the image as a compact mass of greater brightness. Yellow arrow indicated the filamentous projections of EPS-matrix. Scale bar of CLSM images (A–C) = 17.29 µm, and of LTSEM images: (A) = 10 µm, (E and F) = 5 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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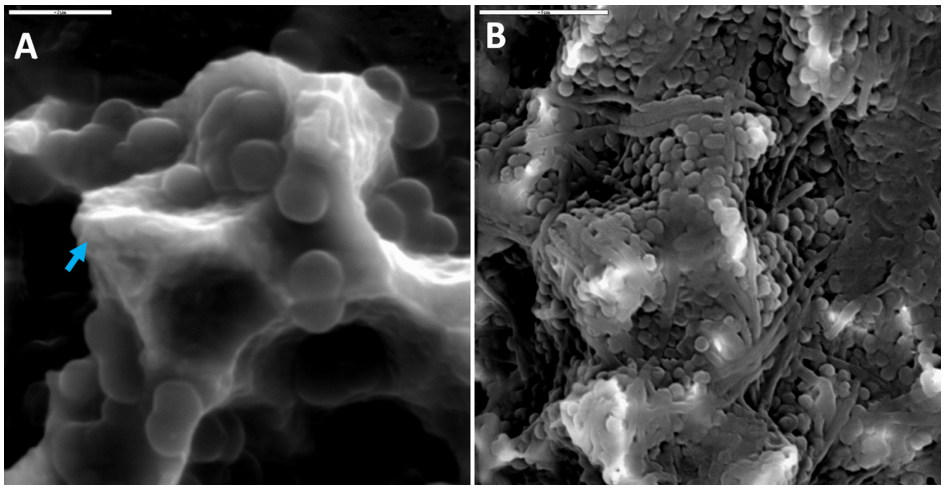

Fig. 2 – LTSEM images of biofilm structure on titanium discs after (A) 12 h and (B) 24 h of incubation. Image (A) shows EPS matrix as a dense mass packing bacterial cells to set up the biofilm, which recognized in the image as a compact mass of greater brightness (blue arrow); scale bar = 2 μm. Image (B), a close up image shows the biofilm, consist primarily of a larger stacks (outward growing masses of bacterial cells), demonstrated the presence of broad channels within the structure; scale bar = 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the three surfaces more complex and the EPS matrix became thicker. In the hydroxyapatite surfaces, clusters of micro-colonies forming a continuous layer of cells were observed protruding the EPS matrix. In the titanium surfaces, larger stacks (outward growing masses of bacterial cells) were identified (Fig. 2B), demonstrating the presence of broad channels within the "towers". On the zirconium surfaces, the biofilms were more fragile, depicting hollow areas between clusters of bacteria. When comparing biofilms thickness after 48 h of development (Table 2), statistically significant differences were observed when comparing hydroxyapatite with titanium or zirconium surfaces ($p < 0.05$, in both cases) as well as between titanium and zirconium surfaces ($p < 0.05$), with biofilm on hydroxyapatite surfaces being significantly thicker than on titanium and hydroxyapatite surfaces. Conversely, no significant differences were observed among the surfaces in terms of vitality (Table 2).

After 72 h the described differences in the biofilm architecture among the three studied surfaces were still present. On hydroxyapatite surfaces, the biofilm covered the entire disk surface as a flat homogenous layer of cells combined with stacks of bacterial aggregations. Channels were evident and were filled with an amorphous extracellular material (Fig. 3D). On titanium surfaces, the EPS-matrix formed a crater-like architecture, mimicking a honeycomb (Fig. 3E). On the zirconium surfaces, the biofilm had a complex morphology with EPS-matrix strands forming networks with the adhered micro-colonies of bacteria mimicking a cobweb (Fig. 3F). With CLSM, the biofilm thickness showed only statistically significant differences when zirconium biofilms were compared to hydroxyapatite and titanium biofilms ($p < 0.01$, in both cases) (Table 2). With LTSEM the morphological characteristics depicted at 72 h were maintained until 120 h suggesting that the biofilms reached a steady state after 72 h of formation. Similarly, the thickness of the biofilms measured with CLSM did not show statistically significant differences when the 96 and 120 h biofilms were compared with the 72 h-biofilm for the three studied surfaces ($p > 0.05$). The cell vitality, however, decreased in the biofilms in the three groups from 72 h to 120 h of incubation.

Table 2 – Observed changes in biofilm vitality and thickness.

Biofilm sample	12 h ($n = 9$)			48 h ($n = 9$)			72 h ($n = 9$)		
	Ha surface	Tn surface	Zn surface	Ha surface	Tn surface	Zn surface	Ha surface	Tn surface	Zn surface
Thickness (μm)									
Mean	11.6	10.2	7.4	21.2	16.1	8.9	24.8	23.2	11.5
Standard deviation	3.8	2.5	1.8	1.9	3.2	2.8	2.4	7.5	5.6
Vitality (%)									
Mean	87.2	85.6	83.7	93.9	88.5	88.2	73.8	83.7	79.9
Standard deviation	8.5	7.4	8.5	4.0	7.1	6.4	21.3	11.1	12.1

Ha, hydroxyapatite; Tn, titanium; Zn, zirconium.

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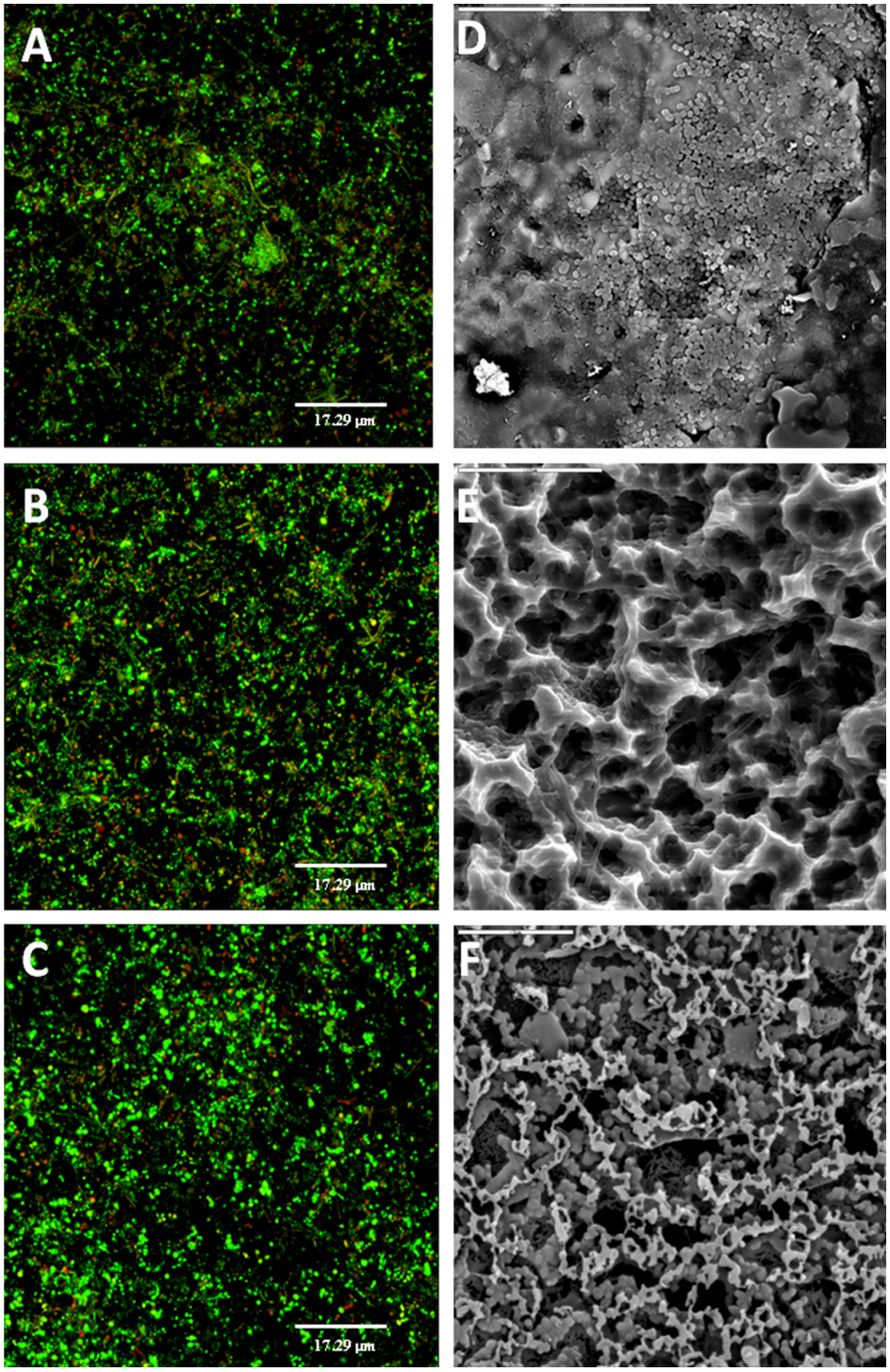

Fig. 3 – Maximum projection CLSM (A–C) and close up LTSEM (D–F) images of biofilm structure on: (A and D) hydroxyapatite, (B and E) titanium and (C and F) zirconium discs after 72 h of incubation. It can be appreciated variations in the architecture of biofilms between materials: biofilms on hydroxyapatite (A and D) cover the disk surfaces with flat homogenous layers of cells combined with bacteria clusters forming stacks, showed channels inside the structure; Biofilms on titanium surface (B and E) acquire a crater-like architecture, with a honeycomb appearance rich in EPS-matrix; Zirconium surface (C and F) biofilms show a cobwebbed appearance, with an amorphous polymeric extracellular matrix surrounding and interconnecting bacteria. Scale bar of CLSM images (A–C) = 17.29 µm. Scale bar of LTSEM images: (D) = 20 µm; (E) = 10 µm and (F) = 5 µm.

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3.2. *Quantitative analysis of biofilms by qPCR*

Fig. 4 shows the kinetic profiles for the six bacterial species (CFU/biofilm) utilized to develop the biofilms on hydroxyapatite, titanium and zirconium surfaces, after being quantified by means of qPCR, at the different incubation times. The numbers (CFU/biofilm) of the different inoculated bacterial species throughout time had similar dynamics in the three studied surfaces. The six bacteria could be detected after 1 h of incubation (Fig. 4), with a predominance of *S. oralis*, *V. parvula* and *A. naeslundii*. *F. nucleatum* and the late colonizers were also detected although in lower amounts. These results are consistent with those observed by CLSM and LTSEM microscopy, where the biomass observed at the first 12 h of development was mainly represented by chains of cocci, corresponding to *S. oralis*, but also by coccobacilli and bacilli, which include *F. nucleatum*. After 24 h of development, the number of primary colonizers continued its increase, while secondary and late colonizers also slowly rose their numbers in the biofilm (Fig. 4). These results correspond with the increase in biomass observed by microscopy at this incubation time. After 72 h of incubation, the biofilms reached their maturity in the three studied surfaces; since the increase in bacterial cells at this stage was not statistically significant in any of the three surfaces at 96 and 120 h (Fig. 4). This observation was consistent with the results observed by microscopy.

Each bacterium (CFU/biofilm) was quantified individually by qPCR in the biofilms formed on the different surfaces, as described ahead:

- When biofilms on hydroxyapatite surfaces at 72 h were compared with the other incubation times used (1, 12, 24, 48, 96 and 120 h), statistically significant differences were observed for all bacteria, except for *S. oralis*. At 72 h, each bacterium reached its maximum quantity, except *S. oralis* that reached its peak at 24 h.
- On titanium surfaces, the amounts of *V. parvula*, *A. actinomycetemcomitans* and *P. gingivalis* demonstrated statistically significant differences when biofilms at 72 h were compared with the rest of incubation times (1, 12, 24, 48, 96 and 120 h). *F. nucleatum* reached its peak at 24 h, for *A. naeslundii* at 48 h and *S. oralis* at 96 h (this last case only demonstrated statistically significant differences compared with those obtained at 1 and 72 h).
- On the zirconium biofilms, *V. parvula*, *A. actinomycetemcomitans* and *P. gingivalis* reached their peak at 72 h and demonstrated significantly higher numbers when compared with the other studied times (1, 12, 24, 48, 96 and 120 h). *F. nucleatum* and *A. naeslundii* achieved highest numbers at 48 h and *S. oralis* at 96 h, with significant differences with the other time points of evaluation.

The comparison among the biofilms formed over the three studied surfaces demonstrated significant differences between biofilms on hydroxyapatite *versus* titanium or zirconium surfaces, but not between titanium and zirconium surfaces. In the early stages, statistically significant differences were observed in the number of *V. parvula* (at 1 h) and *S. oralis* (at 24 h) ($p < 0.01$, in all cases). When biofilms reached its mature state at 72 h, biofilms on hydroxyapatite disks demonstrated larger numbers of total bacteria, doubling the number of bacteria, when compared with the other two biomaterials, for *S. oralis*, *A. naeslundii* and *A. actinomycetemcomitans* (Table 3). No statistically significant differences in the number of bacteria, determined by qPCR, ($p > 0.05$) were observed when comparing titanium and zirconium discs, although the number of total bacteria was slightly greater on zirconium-biofilm, principally due to the presence of primary colonizers: *S. oralis*, *V. parvula*, and *A. naeslundii* (Table 3).

4. Discussion

In this investigation, we compared the structure and bacterial kinetics of an *in vitro* biofilm model developed in three different surfaces: hydroxyapatite, titanium and zirconium. The biofilm model applied, which has been validated in previous studies [21,22,27], is relatively ease to cultivate and assure development of subgingival dental plaque/peri-implant plaque for a realistic and reproducible laboratory simulation of the oral condition, as it has been demonstrated with the present results, since the six inoculated bacteria adhered and matured within the biofilms on the three studied surfaces. The developed biofilm on both implant surfaces, titanium and zirconium, were similar to that formed on a typical tooth surface (hydroxyapatite), in regards to the vitality of bacteria within. The biofilm structure (by CLSM and LTSM), as well as the biofilm kinetics and number of the different bacterial species (by qPCR), was, however, significantly different when comparing hydroxyapatite with both titanium and zirconia surfaces, demonstrating thicker biofilms with higher number of bacteria when reaching the mature state (at 72 h).

There is plenty of evidence in the scientific literature that microbiota around dental implants is influenced by the implant surface and the peri-implant environment, although the bacterial load and the composition are similar to the microbiota around natural teeth [3]. In this investigation, we studied both health and disease associated bacteria inoculated into different surfaces in an *in vitro* biofilm model [5–12]. The selected bacterial species are representative from clusters or complexes associated with periodontal health and disease, as described by Socransky et al. [9] and included initial colonizers as *S. oralis*, intermediate colonizers, belonging to the genus *Actinomyces* or *Veillonella*, and late colonizers including *A. actinomycetemcomitans* and *P. gingivalis*, which are species strongly associated to both periodontitis and peri-implantitis [13,28–31]. This selection was aimed to reproduce the natural dynamics of subgingival biofilm formation with an *in vitro* biofilm model that has been validated both in static as well as in dynamic conditions [21,22].

The structural analysis showed that biofilms were developed on the three tested materials. Different surfaces, however, demonstrated differences in the biofilm tri-dimensional structure even at early stages and these differences were maintained over time. Biofilms on zirconium surfaces were significantly thinner than on titanium and hydroxyapatite surfaces. With LTSEM, the tri-dimensional structure also showed differences both in the deposition of the EPS as well as in the organization of the bacterial cells. In the titanium surfaces,

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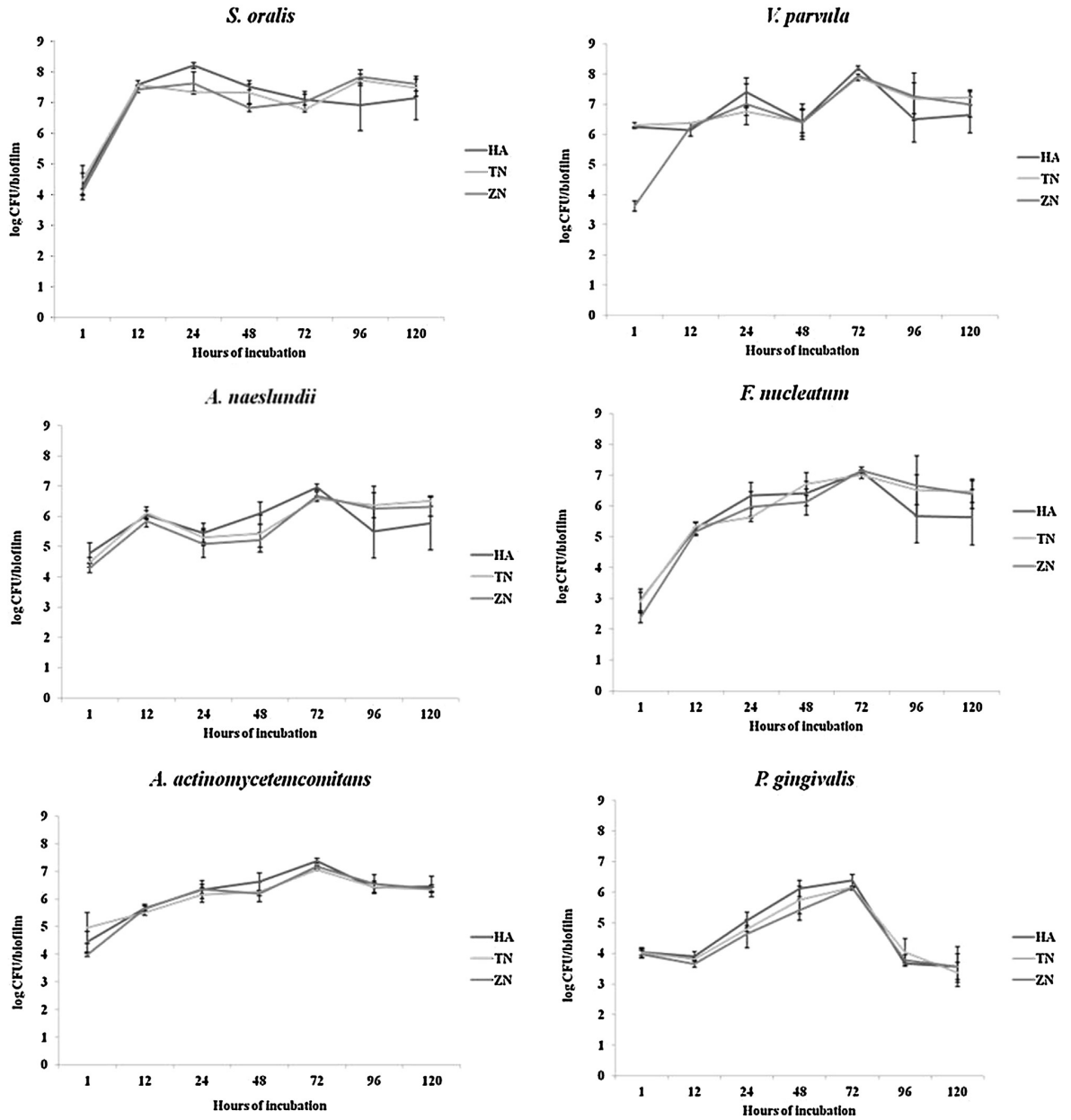

Fig. 4 – Kinetics of incorporation of the six bacterial species in the biofilm (log CFU/biofilm) on each of the materials used in the study: hydroxyapatite (HA), titanium (TN) and zirconium (ZN), obtained by qPCR from biofilms of 1 h to 120 h of incubation, using specific primers and probes directed to the 16S *rRNA* gene.

there was a clear identification of the bacterial stacks and the circulation channels, while on the zirconium surfaces the biofilm adopted a cob-web morphology. Studies using scanning electronic microscopy (SEM) reported similar results when comparing zirconium and titanium surfaces *in vivo* for 24 h. They reported that the percentage of area coverage by biofilm on zirconium material was significantly lower than over titanium surfaces [32]. Schmidlin et al. [20] also using SEM, however, reported a similar biofilm structure when comparing tooth and titanium surfaces.

With the use of qPCR we showed that the biofilm formation and dynamics are similar in the three tested surfaces. After the formation of the acquired pellicle the bacterial attachment by the initial colonizers occurred, followed by cell-to-cell adhesion of secondary and late colonizers. This biofilm-growth dynamics are consistent with the descriptions of biofilm formation on tooth surfaces [2]. One hour after bacterial inoculation, the six species were detected inside the biofilms on the titanium surfaces, which is coincident with the bacterial kinetics described 30–60 min after implant

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Table 3 – Number (CFU/biofilm) of each bacterial species obtained by quantitative real-time polymerase chain reaction (qPCR) in the biofilm of 72 h of develop on the three materials.

Bacteria	Number of each bacteria [CFU/biofilm mean (standard deviation)]		
	Discs material		
	Hydroxyapatite	Titanium	Zirconium
S. oralis	1.2×10^7 (5.5×10^6)	5.9 × 106 (2.1 × 105)	1.3 × 107 (1.1 × 107)
V. parvula	1.5 × 108 (2.5 × 107)	7.8 × 107 (1.9 × 107)	8.7 × 107 (1.1 × 106)
A. naeslundii	9.4 × 106 (2.0 × 106)	3.9 × 106 (8.8 × 105)	4.9 × 106 (1.6 × 106)
F. nucleatum	1.3 × 107 (1.5 × 106)	1.0 × 107 (2.9 × 106)	1.5 × 107 (3.4 × 106)
A. actinomycetemcomitans	2.4 × 107 (4.9 × 106)	1.1 × 107 (7.8 × 105)	1.5 × 107 (1.6 × 106)
P. gingivalis	2.6 × 106 (1.2 × 106)	1.4 × 106 (4.7 × 104)	1.4 × 106 (2.0 × 105)
Total bacteria (cell summation)	2.2 × 108	1.1 × 108	1.4 × 108

installation [5,7,8]. The tested biofilms continued their growth until reaching a plateau with a maturity peak at 72 h in all three tested surfaces, although the number of bacteria was significantly higher in the hydroxyapatite surfaces. This is also coherent with the reports from comparing total bacterial loads between tooth and implant samples [7]. Conversely, no significant differences were found in the number of bacteria when comparing the zirconium and titanium surfaces, suggesting that both surfaces are equally "susceptible" to plaque accumulation.

There are, however, conflicting results in the literature on the possible effects of implant surface topography on plaque formation and maturation. Rimondini et al. [19] studied *in vitro* bacterial adhesion to titanium and two types of zirconium surfaces, and reported that zirconium showed significantly more adherent *Streptococcus mutans* than did titanium after 24 h, while *Streptococcus sanguis* seemed to adhere easily to titanium specimens. No differences were noted for *Actinomyces* spp. Conversely, Lee et al. [17] did not report significant differences in the *in vitro* bacteria adhesion (*S. sanguis*) between titanium and zirconium after 2 h of incubation. Al-Radha et al. [18] observed that *Streptococcus mitis* had less affinity to adhere to zirconium than titanium surfaces after 6 h of *in vitro* biofilm formation. Schmidlin et al. [20] demonstrated, on titanium surfaces similar kinetics to what it is reported in the present study, with an initial adherence for *S. oralis*, *A. naeslundii*, *F. nucleatum* and *Veillonella* species, although there are differences in the reported amounts for each bacteria (log CFU/biofilm), due, perhaps, to different model conditions and assessing methods. With our model, it was observed that the initial colonizers *S. oralis, A. naeslundii* and *V. parvula*, did not differ significantly between the biofilms on titanium and zirconium at 1, 12 or 24 h, which is in agreement with Rimondini et al. [19], who showed no significant differences in bacterial adhesion of *Actinomyces* spp. within the first 24 h. All these studies, however, are short-term evaluations (24 or less hours) and hence, only study early bacterial adhesion.

The obtained results on mature biofilms are also in accordance with the results reported by de Oliveira et al. [28] using qPCR. They showed no statistically significant differences between DNA copy numbers of *A. actinomycetemcomitans*, *P. gingivalis* and total bacteria for both zirconium and titanium surfaces *in vivo*. Similarly, Rimondini et al. [19] showed no significant differences in the early *in vitro* colonization of *P. gingivalis* (24 h). Quirynen et al. (1994), however, postulated that biomaterials with lower surface free energy, such as zirconium, accumulated more coccoid microorganisms and less pathogenic species, based in differential phase-contrast microscopy [33]. These results are in agreement with our results in mature biofilm on zirconium surfaces, where the quantity of *S. oralis* was higher than in the titanium-surfaces.

In summary, while the formation and dynamics of this *in vitro* biofilm model was similar, irrespective of the surface of inoculation (hydroxyapatite, titanium or zirconium), there were significant differences in regards to the biofilm thickness and three-dimensional structure. The clinical implications of this finding have to be evaluated but it may be hypothesized that the reported differences could have an impact in the biofilms susceptibility to antimicrobial agents, which could affect both preventive measures (*e.g.* the use of antiseptics as part of oral hygiene procedures) and treatment approaches (*e.g.* local or systemic antimicrobials). In addition, surfaces more prone to harbor more complex biofilms may be more susceptible for the development of peri-implant diseases.

5. Conclusions

In conclusion, this investigation has demonstrated that the formation and dynamics of an *in vitro* biofilm model was similar, irrespective of the surface of inoculation (hydroxyapatite, titanium or zirconium). There were significant differences, however, between the biofilms on hydroxyapatite, on one side, and those on titanium and zirconium surfaces, on the other side, in respect to the three dimensional organization of the biofilms and on the number of bacteria within the biofilms. This investigation has also shown that the use of CLSM, LTSEM and qPCR allows the study of *in vitro* biofilm models, both in terms of structure and morphology, as well as bacterial dynamics and kinetics.

Acknowledgements

This study was funded by CDTI (Center for the Development of Industrial Technology) of the Spanish Ministry of Science and Innovation and the company Dentaid (Barcelona, Spain), contract number 332/2011.

We thank Institut Straumann AG (Basel, Switzerland) for the supplemented material, and we thank, for their technical assistanc, A. Cortés and LM. Alonso (Center of Microscopy and Cytometry, University Complutense, Madrid, Spain) and Fernando Pinto (ICA Microscopy Service, CSIC, Madrid, Spain).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dental.2014.07.008.

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2 Estudio 2

Fernández E, Sánchez MC, Llama-Palacios A, Sanz M, Herrera D. Antibacterial effects of toothpastes evaluated in an *in vitro* biofilm model. *Oral Health and Preventive Dentistry* (2016). (Aceptado para publicación).

RESUMEN

Objetivos: Comparar el efecto antibacteriano de diferentes dentífricos en un modelo *in vitro* de biofilm oral, empleando el método "slurry" para la aplicación de los dentífricos

Material y Método: Se emplearon cuatro dentífricos comerciales, de los cuales, dos contenían fluoruro de sodio (NaF) (1,450 y 2,500 ppm) y dos NaF con triclosán o fluoruro de estaño y un control negativo (NC). Las bacterias crecieron en biofilm sobre discos de hidroxiapatita durante 72 horas y fueron expuestos durante dos minutos a los dentífricos en forma de "slurry" o al NC. Los biofilms fueron analizados mediante la reacción en cadena de la polimerasa a tiempo real (qPCR), combinada con propidio monoazida (PMA). Las comparaciones se realizaron mediante ANOVA y el test t de Student con la corrección de Bonferroni para comparaciones múltiples.

Resultados: El dentífrico que contenía NaF y fluoruro de estaño demostró una actividad antimicrobiana superior para *A. actinomycetencomitans*, *P. gingivalis* y *F. nucleatum* al compararlo con los dentífricos que contenían NaF y triclosan, 1,450 ó 2,500 NaF.

Conclusión: El protocolo propuesto para la evaluación de los dentífricos en forma de "slurries" en un modelo de biofilm es una metodología adecuada para comparar el efecto antimicrobiano *in vitro* de los diferentes dentífricos.

Antibacterial effects of toothpastes evaluated in an *in vitro* biofilm model

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Article history:

Received 29 July 2015

Received in revised form 12 December 2015

Accepted 25 December 2015

Keywords:

Sodium Fluoride
Triclosan
Stannous Fluoride
Oral biofilm
qPCR
Slurry
Toothpaste

Abstract

Objectives: To test the antibacterial effects of different toothpastes in an *in vitro* oral biofilm model including relevant periodontal pathogens, with the slurry method for toothpaste application.

Material and methods: Four commercially available toothpastes, two containing sodium fluoride (NaF) at different concentrations (1,450 and 2,500 ppm), two NaF plus either triclosan or stannous fluoride and a control phosphate buffered saline (PBS) were used. Multispecies biofilms containing 6 oral bacteria were grown on hydroxyapatite discs for 72 hours and then exposed for 2 minutes to the toothpaste slurries or phosphate buffer saline (PBS) by immersion in continuous agitation at 37ºC. Biofilms were then analyzed by means of real-time polymerase chain reaction (PCR), combined with propidium monoazide (PMA). Statistical evaluation included the use of ANOVA and Student t-test, with Bonferroni correction for multiple comparisons.

***Results*:** The toothpaste containing NaF and stannous fluoride demonstrated superior antimicrobial activity for *A. actinomycetencomitans, P. gingivalis* and *F. nucleatum* when compared to those containing NaF and triclosan, 1,450 ppm NaF or 2,500 NaF, in this multispecies biofilm model.

Conclusion: The proposed model, for the evaluation of toothpastes in the format of slurries, was able to detect significant differences in the antimicrobial effects among the tested NaF containing toothpastes, with the stannous fluoride- based formulation achieving better results. The use of toothpaste as slurries and real-time PCR, with PMA is an adequate methodology to compare the *in vitro* antimicrobial effect of different toothpastes.

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Introduction

Oral diseases, such as dental caries and periodontitis, represent a serious public health problem worldwide (1). Both diseases have in common their bacterial etiology, with causative microorganisms being organized as multispecies biofilms, “dental plaque” (2). Even though more than 700 bacterial species can be found in the oral cavity, specific groups of these microorganism have been identified as significantly associated with periodontal diseases, including gingivitis and periodontitis (3). Current knowledge on the significant associations between periodontal and systemic diseases highlight the importance of prevention of these oral biofilm related diseases that is mainly based on oral hygiene practices aimed for plaque control (4, 5).

The American Dental Association (ADA) recommends as the most efficient oral hygiene method tooth brushing for two minutes twice a day, combined with the use of dental floss once a day. Tooth brushing is the most effective method since it combines the mechanical effect with the mechanism to deliver toothpaste on tooth surfaces. However this effectiveness depends on the skill of the individuals and their personal motivation (6). Nevertheless, numerous studies have suggested that the combined use of mechanical (brushing) and chemical (toothpaste and/or mouth rinse) therapy is the most effective way of achieving the desired control of dental plaque (7).

The toothpaste is an ideal vehicle to carry antibacterial substances. These chemotherapeutic products must be safe, effective in reducing plaque and gingivitis, must have substantivity, should impact the pathogenic flora and have a pleasant taste (8). Fluorides have demonstrated efficacy in caries prevention, while antiplaque agents, such as chlorhexidine, cetylpyridinium chloride, essential oils, stannous fluoride and triclosan, among others, have demonstrated antiplaque and antigingivitis effects (9, 10).

In vitro testing of antibacterial agents may provide interesting insights into their potential clinical efficacy. Agents with demonstrable *in vitro* antibacterial activity may be effective against the same microorganisms *in vivo*, whereas

agents without demonstrable *in vitro* antibacterial activity are unlikely to exhibit *in vivo* antibacterial activity (11). However, the vast majority of *in vitro* studies with toothpastes have been performed on isolated bacterial species instead on subgingival biofilm models that include the main periodontal pathogens. It is well established that bacteria organized in biofilms are more resistant than bacteria in planktonic growth against antimicrobial agents (12-14), what makes more relevant to evaluate the antimicrobial activity of toothpastes in biofilm models. Furthermore, while there are numerous *in vitro* and *in vivo* studies evaluating the antimicrobial effects by mouth rinses, toothpastes are more difficult to assess, and less studies are available.

For evaluating the antimicrobial activity of toothpastes the slurry method has been proposed, for example, for testing tooth paste activity with a young (16 hours) subgingival biofilm model (15), used *in vivo* models such as the plaque regrowth model (16-20), or for a combination of *in vitro* and *ex vivo* study, evaluating the Minimum Inhibitory Dentifrice Concentrations for oral and non-oral microorganisms (11). Most of these studies used culture-based techniques to evaluate the antimicrobial effect of the toothpastes, although the limitations of these techniques are well known in terms of sensitivity, specificity and dependence on well-trained personnel (21).

Our research group has developed and tested an *in vitro* subgingival biofilm model which contains initial (*Streptococcus oralis* and *Actinomyces naeslundii*), early (*Veillonella parvula)* and secondary (*Fusobacterium nucleatum*) colonizers, as well as two well-known periodontal pathogens, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, thus simulating the *in vivo* composition of the subgingival microbiota (22). Using this biofilm model, our research group has recently tested the use of combined molecular techniques to study the antimicrobial effects of different substances when applied to oral biofilms in order to overcome some of the limitations of the culture techniques (23). This method combines bacterial quantification through real-time polymerase chain reaction (qPCR)

and the dye propidium monoazide (PMA), which discriminates between live and dead bacteria. The use of molecular techniques offers clear advantages: PCR-based techniques may enhance specificity and sensitivity over traditional culture techniques, as well as their ability to obtain results more rapidly (24); however, a major disadvantage of PCR is that it detects DNA from both viable and dead bacterial cells, due to the relatively long persistence of DNA after cell death (25-28). To avoid this disadvantage, the qPCR technique was combined with PMA, which only can penetrate into bacterial cells with compromised membrane integrity, resulting in the capability to distinguish between viable and damaged cells by PCR (27-29).

It was, therefore, the purpose of the present investigation was to compare the antibacterial effects of toothpastes, by developing a new methodology, which used the slurry method for applying the toothpaste on a multispecies subgingival *in vitro* oral biofilm model and the evaluation of its efficacy by qPCR and PMA.

Material and methods

Bacterial strains and culture conditions

Standard reference strains of *S. oralis* CECT4 907T, *V. parvula* NCTC2 11810, *A. naeslundii* ATCC3 19039, *F. nucleatum* DSMZ1 20482, *A. actinomycetemcomitans* DSMZ 8324 and *P. gingivalis* ATCC 33277 were used. Bacteria were grown on blood agar plates (Oxoid no. 2; Oxoid Ltd., Basingstoke, UK), supplemented with 5% (v/v) sterile horse blood (Oxoid), 5 mg/L hemin (Sigma, St Louis, MO, USA) and 1.0 mg/L menadione (Merck, Darmstadt, Germany) in anaerobic conditions 10% H_2, 10% of $CO_2$and balance N_2) at 37º C for 72 h.

Biofilm development

Biofilms were developed as previously described (22). In brief, pure cultures were grown anaerobically in a protein rich medium containing brain-heart infusion (BHI) (Becton, Dickinson and Company, USA) supplemented with 2.5 g/L mucin (Oxoid), 1.0 g/L yeast extract (Oxoid), 0.1 g/L cysteine (Sigma), 2.0 g/L sodium bicarbonate (Merck), 5.0 mg/mL hemin (Sigma), 1.0 mg/L menadione (Merck) and 0.25% (v/v)

glutamic acid (Sigma). The bacterial growth was measured by spectrophotometry and a mid-exponential phase a bacterial mixed was prepared containing 10^3 colony forming units (CFU)/mL for *S. oralis*, 10^5 CFU/mL for *V. parvula* and *A. naeslundii*, and 10^6 CFU/mL for *F. nucleatum*, *A. actinomycetemcomitans* and *P. gingivalis*.

Sterile calcium hydroxyapatite discs of 7 mm of diameter and 1.8 mm of thickness (Clarkson Chromatography Products, Williamsport, PA, USA) were placed in the wells of a 24-well tissue culture plate (Greiner Bio-one, Frickenhausen, Germany). Each well was inoculated with 1.5 mL pooled bacteria culture prepared and incubated in anaerobic conditions (10% H_2, 10% CO_2, and balance N_2) at 37ºC for 72 h. To ensure sterile conditions, medium without bacteria inoculum were included as negative control.

Tested toothpastes

The following commercially available, sodium fluoride-containing toothpastes were evaluated: 0.3% triclosan with polyvinylmethil ether and maleic acid copolymer (PVM/MA) and sodium fluoride (1,450 ppm) (TC) (Colgate Total Original®; Colgate-Palmolive, Madrid, Spain); stannous fluoride (1,100 ppm), with sodium hexametaphosphate (SHMP) and sodium fluoride (350 ppm) (SnF) (Oral B Pro-Expert; Procter & Gamble UK, Weybridge, Surrey, UK); 0.32% sodium fluoride (1,450 ppm) (1.45NaF) (Colgate Total Pro-Interdental; Colgate-Palmolive), sodium fluoride at 2,500 ppm (2.5NaF) (Fluor-Aid 250; Dentaid S.L., Cerdanyola, Spain). Phosphate buffered saline (PBS; pH 7.4), served as the negative control.

Exposure of biofilms to toothpastes

Mature biofilms (72 h of incubation) were gently washed with PBS to detach non-adhered cells and to remove the culture medium. To evaluate the bactericidal effects of the tested products, 750 µL of the toothpastes prepared as slurry, and PBS as negative control, were placed in the wells of a 24-well tissue culture plate (Greiner Bio-one) and biofilms immersed. The toothpaste slurries were prepared dissolving 0.5 g of the toothpastes in 1 mL of sterile PBS and vortexing until their complete dilution (with times ranging between 1-5 min due to the

different density of each dentifrice). The slurries were applied with a single exposure of 2 min at 37ºC with a continuous agitation at 90 rpm in order to mimic brushing movements. Then, the biofilms were sequentially washed, with PBS, three times, for 10 s each to eliminate the antimicrobial product. A single exposure of the biofilms to the toothpaste slurries was selected, in order to simulate a single episode of tooth brushing.

The experiments were repeated three times, in different days, with trios of samples and with fresh bacterial cultures. In each experiment, the four toothpastes and the PBS were tested together.

PMA treatment

After the antimicrobial treatment, biofilms were disrupted by vortexing for 2 min, in 1 mL of PBS. Immediately after the antimicrobial action, the DNA-intercalating dye PMA was used with the method previously reported (23). In brief, PMA (Biotium Inc., Hayword, CA, USA) was added at final concentrations of 100 µM to sample tubes containing 250µL of disaggregated biofilm cells. Following an incubation period of 10 min at 4ºC in the dark, the samples were subjected to photo-induced cross-linking of PMA by light exposing for 30 min using a 550 W halogen light source, placed 20 cm above the samples. The sample tubes were laid down horizontally on ice during this period to avoid excessive heating. After photo-induced cross-linking, the cells were centrifuged at 12,000 rpm for 3 min prior to DNA isolation. To control for any influence on the bacteria viability of the process alone (incubation at 4ºC and exposure to light source), 250 µL of disaggregated biofilm cells (all derived from the same disaggregated biofilm cell suspension) subjected to the same process, but without the exposure to PMA, were used as control samples.

DNA isolation and qPCR

The DNA was then isolated from all samples using a commercial kit (ATP Genomic DNA Mini Kit®; ATP biotech, Taipei, Taiwan), following manufacturer´s instructions. The hydrolysis probes 5´nuclease assay PCR method was used for detecting and quantifying the bacterial DNA. Primers [synthesized by Life Technologies

Invitrogen (Invitrogen, Carlsbad, CA, USA)] and probes [synthesized by Life Technologies Applied Biosystems (Applied Biosystems, Carlsbad, CA, USA)] sequences, targeted against 16S rRNA gene, are shown in Table 1. The qPCR amplification mixtures contained 10 µL of 2x master mixture (LC 480 Probes Master; Roche Diagnostic GmbH, Mannheim, Germany), optimal concentrations of primers and probe (respectively, 300, 300 and 200 nM for *A. actinomycetemcomitans*; 300, 300 and 300 nM, for *P. gingivalis* and 600, 600 and 300 nM for *F. nucleatum*), and 5 µL of DNA from samples. The negative control was 5 µL of sterile water [no template control (NTC)] (Water PCR grade, Roche). The samples were subjected to an initial amplification cycle of 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Analyses was performed with a LightCycler® 480 II thermocycler (Roche). The plates used in the study were FramStar 480 of natural frame and white wells (4titude; The North Barn; Damphurst Lane, UK), sealed by QPCR Adhesive Clear Seals (4titude).

Each DNA sample was analyzed in duplicate. Quantification cycle (Cq) values, previously known as cycle threshold (Ct) values, describing the PCR cycle number at which fluorescence rises above the baseline, were determined using the provided software package (Roche).

Quantification of viable cells by PMA-qPCR

Quantification of viable cells by qPCR was based on standard curves. One mL of viable *P. gingivalis*, *A. actinomycetemcomitans* and *F. nucleatum* cell suspensions, containing 10^{10} CFU/mL (determined by optical density at 550 nm, based on the growth curves previously generated, and confirmed by plating 100 µL of diluted serially aliquots on supplemented blood agar plates at 37°C for 4-7 days, in anaerobic conditions) were treated with PMA (following the protocol described above). Cultures were subjected to DNA isolation using the kit ATP Genomic DNA Mini Kit® (ATP biotech), following manufacturer's instructions. The DNA concentration was measured on a Nanodrop® ND-

1000 Spectrophotometer (Nanodrop, Wilmington, DE, USA). Serial dilutions of each DNA were performed on sterile water (Water PCR grade, Roche), with a range 10^{10}-10 CFU/mL correspondence, and subjected to the qPCR assay described above. Standard curves were constructed by plotting Cq values generated from qPCR against *P. gingivalis*, *A. actinomycetemcomitans* and *F. nucleatum* (log CFU/mL). The correlation between Cq values and CFU/mL were automatically generated through the LC480 Software® 1.5 (Roche).

All assays were developed with a linear quantitative detection range established by the slope range of 3.2-3.3 cycles/log decade, r^2>0.977 and an efficiency range of 1.9-2.0. Values below this linear quantitative detection range may be detectable but not quantifiable, since lower limits of quantification are poorly defined.

Measures to avoid carryover DNA were established. In spite of this, when NTC was detectable, the limit of detection (LOD) was established on the last value of the standard curve that holds 5 cycles of difference with NTC.

Data analyses

Results were expressed as CFU/mL. Counts of the three tested bacterial species then were log transformed to achieve a normal distribution (evaluated through skewness and kurtosis). Two analyses were carried out: firstly, every test group was compared individually to the untreated group by means of Student t-test, in order to evaluate the impact of each product. Secondly, all groups were evaluated together, to detect significant differences among the five groups, by means of ANOVA with the Multiple Rank Test (MRT) as the post hoc test, in order to detect differences between specific groups. Both analyses were performed individually for each selected bacterial species.

The level of significance was established in $p<0.05$. Since four comparisons were performed when using t-test, the Bonferroni correction was used, and the level of significance was divided by the number of comparisons (plus one), leading to a threshold for significance of $p<0.01$.

Results

The results of the immediate effect of a single exposure to the toothpastes on the multispecies oral biofilm model evaluated by qPCR and expressed as log of CFU/mL, are graphically presented in Fig. 1.

For *A. actinomycetemcomitans* (Table 2), significant differences among groups were observed ($p<0.001$). The higher values corresponded (according to the MRT) to PBS, and the lower to SnF containing toothpastes. When assessed by paired comparisons with the negative control, significant reductions were observed for SnF (89.2% of reduction, $p<0.001$), TC (87.0%, $p=0.006$) and 1.45NaF (69.8%, $p=0.005$).

For *F. nucleatum* (Table 2), the overall comparison among groups showed statistically significant differences ($p=0.001$), which corresponded (MRT) to lower counts for SnF and 2.5NaF as compared to the negative control; lower counts of SnF when compared to TC and 1.45NaF; and lower counts of 2.5NaF versus 1.45NaF. Paired comparisons with the negative control demonstrated significant differences for SnF (89.6% of reduction, $p<0.001$) and 2.5NaF (83.2%, $p=0.008$).

For *P. gingivalis* (Table 2), significant differences were detected among groups ($p<0.001$), identified (MRT) as differences between the negative control and the four dentifrices. Additional differences were detected also for SnF, with lower counts than the other three test groups. TC showed lower values than 1.45NaF; and 2.5NaF lower than 1.45NaF. With regards to the pair comparisons of the test groups with the negative control, all the comparisons were statistically significant (for TC, 84.7% of reduction, $p<0.001$; for SnF, 97.0%, $p<0.001$; for 2.5NaF, 92.5%, $p<0.001$; for 1.45NaF, 66.2%, $p=0.003$).

Discussion

The results of this investigation have shown that the use of toothpaste slurries applied in a multispecies oral biofilm model was able to detect differences, not only between the negative control and the tested toothpastes, but also among the active agents, in terms of counts of some of the most relevant periodontal pathogens, namely *P. gingivalis*, *A. actinomycetemcomitans* and *F. nucleatum*. The two first mentioned

have shown a strong association to periodontitis (30), while the third plays an important role in biofilm development, building the scaffold between the early colonizers and the true oral pathogens (31). A single 2-minute exposure of the tested toothpastes to a mature oral biofilm in continuous agitation to mimic *in vivo* brushing effect resulted in a bactericidal effect, demonstrating a significant reduction in the viable microbial load when compared to the negative control. This was true for both toothpastes with recognised antiplaque agents (SnF, TC), but also for fluoride toothpastes (1.45NaF, 2.5NaF), showing an antibacterial effect of fluoride toothpastes.

The main goal of the present research was not to identify a selective inhibitory effect of the tested toothpastes, it was to develop an effective method to compare the antibacterial effect of different toothpastes on three bacterial species strongly associated with periodontitis. The proposed methodology was able to detect differences between the tested formulations: e.g. SnF was significantly more effective than the other three toothpastes for *P. gingivalis*, than TC and 1.45NaF for *F. nucleatum*, and than 1.45NaF and 2.5NaF for *A. actinomycetemcomitans*. Significant antimicrobial effects of stannous fluoride have been also reported in others *in vivo* studies (16), showing that stannous fluoride was superior to conventional fluoride toothpastes in terms of plaque inhibition. In addition, the antiplaque and antigingivitis efficacy of commercially available stannous fluoride-containing toothpastes have been established in 6-month home-use randomized clinical trials (RCTs) (10), which provides the highest level of evidence in the evaluation of oral hygiene products. Some of the available RCTs have tested the most recent formulation, including SHMP.

Another relevant finding of the present study, in the comparisons among toothpastes, was that 2.5NaF was significantly better than 1.45NaF for *F. nucleatum* and *P. gingivalis*. Fluoride is the most important active agent in toothpastes, combined with the mechanical toothbrushing, on dental caries prevention (32). Its effects are concentration dependent (33), which is

confirmed with the results of the present *in vitro* study.

Another relevant finding was the limited *in vitro* effects of TC, as compared to the other tested formulations, since it was only more effective than 1.45NaF for *P. gingivalis*. Triclosan is a non-ionic, bisphenol and germicidal agent, with low toxicity and broad-spectrum activity (against both Gram-positive and Gram-negative bacteria) (34). When combined with PVM/MA, triclosan may keep antimicrobial activity for up to 12 h after brushing. Scientific evidence, based on home-use, 6-month RCTs is available to support the antiplaque and antigingivitis effects of toothpaste with triclosan and PVM/MA, although with significant heterogeneity (10). The lack of agreement between the *in vitro* results of the present study and the results of 6- month RCTs may be associated to the suggested anti-inflammatory effects of the triclosan molecule (not relevant for this model) (35) and the limited relevance, in *in vitro* models, of the presence of PVM/MA in the formulation.

The effects of SnF and TC have been directly compared *in vivo* in a 6-month RCT (36) and better results were found for SnF compared to TC, in terms of gingival index and gingival bleeding. Conversely, other clinical studies reported better results for TC for plaque and gingival indices, both in 24-hour (37) 6-week (38, 39) or 6-month (40) studies. Finally, other report was not able to detect differences between both products, under non- brushing conditions (17).

The difficulties to evaluate the effectiveness of toothpastes without the variability associated to tooth brushing, led to the proposal to prepare slurries of dentifrices, to use them as a rinse, avoiding the need to brush. This technique has been applied previously in different studies, under non-brushing conditions (16-20), in an *in vitro* study (15) or in an *in vitro* and *ex vivo* study (11). These studies tested the different toothpastes converted into slurries, using them as mouth rinses and under non-brushing conditions. The present study confirmed the validity of the slurry method to compare toothpastes in an *in vitro* biofilm model, even comparing with other *in vitro* and *in vivo* studies,

achieving similar results.

In addition, the validity of culture-independent molecular methods to assess the antimicrobial effects of antiplaque agents was demonstrated in the present study, since a PCR-based method was able to accurately detect and quantify viable bacteria after the antimicrobial treatment. This PMA-qPCR method was tested previously in our laboratory with the proposed *in vitro* biofilm model, showing a clear distinction between DNA from viable and dead cells (23).

Within the limitations of using a simplified *in vitro* model, it can be concluded the proposed model was able to detect significant differences in the antimicrobial effects of the tested sodium-fluoride containing toothpastes, which were significantly better for the stannous fluoride-based toothpaste when compared to the other tested toothpastes. The antimicrobial effects of toothpastes, in the format of slurries, can be compared in an *in vitro* multispecies biofilm model, using culture-independent microbiological techniques.

Conflict of interest statement

The authors declare that they do not have any conflict of interest in relation to this investigation.

Table 1. Primers and probes used for quantification of genomic DNA from the target bacteria. Primers and probes were targeted against 16S rRNA gene.

Bacteria	Sequence (5´-3´)	Length (bp)	Reference
Pg Forward Reverse Probe	 GCGCTCAACGTTCAGCC CACGAATTCCGCCTGC 6FAM-CACTGAACTCAAGCCCGGCAGTTTCAA-TAMRA	67	(41)
Aa Forward Reverse Probe	 GAACCTTAC CTACTCTTGACATCCGAA TGCAGCACCTGTCTCAAAGC 6FAM-AGAACTCAGAGATGGGTTTGTGCCTTAGGG-TAMRA	80	(42)
Fn Forward Reverse Probe	 GGATTTATTGGGCGTAAAGC GGCATTCCTACAAATATCTACGAA 6FAM-CTCTACACTTGTAGTTCCG-TAMRA	162	(42)

bp= base pairs

Pg, P. gingivalis; *Aa, A. actinomycetemcomitans*; *Fn*, *F. nucleatum*

Table 2. Comparison of the effect of the tested toothpastes on cell vitality of *P. gingivalis*, *A. actinomycetemcomitans* and *F. nucleatum* in a mature biofilm, obtained by qPCR. All samples were treated with propidium monoazide before DNA extraction. Student t-test was used to compare the effect between each test group and the control; and ANOVA, with multiple rank test (MRT) for the overall comparison among groups.

A. actinomycetemcomitans					
Treatment	Untreated (UN, n=9)	TC (n=9)	SnF (n=9)	1.45NaF (n=9)	2.5NaF (n=9)
Mean (log CFU/mL)	6.50	5.55	5.12	5.74	5.99
SD (log CFU/mL)	0.34	0.82	0.60	0.60	0.54
t test*	reference	**0.0059**	**0.0000**	**0.0048**	0.0305
ANOVA*	**0.0004**				
MRT	UN versus TC, SnF, 1.45NaF		SnF versus 1.45NaF, 2.5NaF		

F. nucleatum					
Treatment	Untreated (UN, n=9)	TC (n=9)	SnF (n=9)	1.45NaF (n=9)	2.5NaF (n=9)
Mean (log CFU/mL)	6.12	5.61	4.97	6.02	5.37
SD (log CFU/mL)	0.57	0.47	0.58	0.83	0.47
t test* (p value)	reference	0.0522	**0.0006**	0.7701	**0.0077**
ANOVA* (p value)	**0.0011**				
MRT	UN versus SnF, 2.5NaF	TC versus SnF	SnF versus 1.45NaF, 2.5NaF	1.45NaF versus 2.5NaF	

P. gingivalis					
Treatment	Untreated (UN, n=9)	TC (n=9)	SnF (n=9)	1.45NaF (n=9)	2.5NaF (n=9)
Mean (log CFU/mL)	6.62	5.60	5.04	6.00	5.46
SD (log CFU/mL)	0.24	0.47	0.36	0.47	0.29
t test* (p value)	reference	**0.0000**	**0.0000**	**0.0031**	**0.0000**
ANOVA* (p value)	**0.0000**				
MRT	UN versus TC, SnF, 1.45NaF, 2.5NaF	TC versus SnF, 1.45NaF	SnF versus 1.45NaF, 2.5NaF	1.45NaF versus 2.5NaF	

*In bold, statistically significant differences: for t-test, the threshold was p<0.01 and for ANOVA p<0.05.

UN: phosphate buffered saline (PBS), served as the negative control; TC: 0.3% triclosan with polyvinylmethil ether and maleic acid copolymer (PVM/MA) and sodium fluoride (1,450 ppm); SnF: stannous fluoride (1,100 ppm), with sodium hexametaphosphate (SHMP) and sodium fluoride (350 ppm); 1.45NaF, 0.32% sodium fluoride (1,450 ppm); and 2.5NaF, sodium fluoride at 2,500 ppm.

SD: standard deviation; CFU/mL, colony forming units per mL.

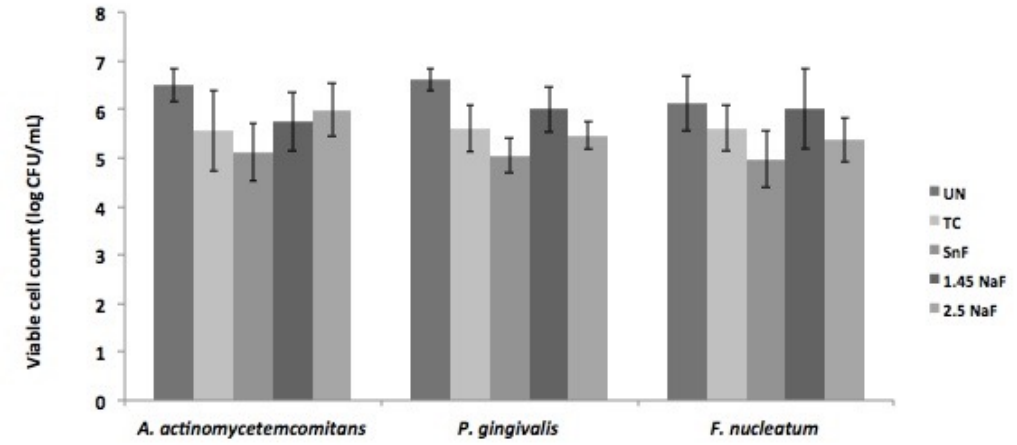

Figure 1. Graphical representation showing viable cell counts (medium and DS), as log of colony-forming units per mL, (log CFU/mL) for *P. gingivalis*, *A. actinomycetemcomitans* and *F. nucleatum* in *in vitro* subgingival biofilms (n=9) after treatment with the tested toothpastes. [UN: phosphate buffered saline (PBS), served as the negative control; TC: 0.3% triclosan with polyvinylmethil ether and maleic acid copolymer (PVM/MA) and sodium fluoride (1,450 ppm); SnF: stannous fluoride (1,100 ppm), with sodium hexametaphosphate (SHMP) and sodium fluoride (350 ppm); 1.45NaF, 0.32% sodium fluoride (1,450 ppm); and 2.5NaF, sodium fluoride at 2,500 ppm].

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3 Estudio 3

Sánchez MC, Fernández E, Llama-Palacios A, Figuero E, Herrera D, Sanz M. Response to antiseptic agents of periodontal pathogens in in vitro biofilms on titanium and zirconium surfaces. *Dental Materials* (2017) Feb 22. pii: S0109-5641(16)30523-1. doi: 10.1016/j.dental.2017.01.013. [Epub ahead of print].

RESUMEN

Objetivos: El objetivo de este estudio fue desarrollar biofilms *in vitro* sobre superficies SLA de titanio (Ti-SLA) y zirconio (ZrO_2) y evaluar el efecto de agentes antisépticos en el número de patógenos periodontales.

Material y Métodos: Se desarrolló un biofilm *in vitro* sobre discos estériles de Ti-SLA y ZrO_2. Se aplicaron tres agentes antisépticos [clorhexidina y cloruro de cetil piridinio (CHX/CPC), aceites esenciales (EEOOs) y cloruro de cetil piridinio (CPC)] al biofilm de 72 horas, sumergiendo los discos durante 1 minuto en la solución antiséptica, tanto en condiciones estáticas como dinámicas. Las células vivas [unidades formadoras de colonias (CFU/mL)] se cuantificaron a través de la reacción en cadena de la polimerasa en modalidad cuantitativa (qPCR) combinada con propidio monoazida (PMA). Se estableció un modelo para determinar el efecto de los agentes sobre las células bacterianas de *A. actinomycetemcomitans*, *P. gingivalis* y *F. nucleatum* en cada superficie.

Resultados: La exposición a cada una de las soluciones antisépticas dio como resultado una reducción estadísticamente significativa en el número de especies bacterianas incluidas en el biofilm multiespecies *in vitro*, tanto en la superficie Ti-SLA como en ZrO_2

(p<0.001), la cual fue de más de dos órdenes para *A. actinomycetemcomitans,* para *P. gingivalis* dos órdenes en Ti-SLA y más de tres órdenes sobre ZrO_2, para *F. nucleatum* más de cuatro órdenes. No se encontraron diferencias estadísticamente significativas en el recuento de las bacterias testadas entre los biofilms *in vitro* formados sobre Ti-SLA y ZrO_2, tras la exposición a los agentes antimicrobianos bajo condiciones estáticas o dinámicas.

Conclusión: *A. actinomycetemcomitans*, *P. gingivalis* y *F. nucleatum* respondieron de manera similar a la exposición de antisépticos al crecer en biofilms multiespecies sobre superficies de titanio y zirconio, a pesar de las diferencias estructurales entre estas comunidades bacterianas.

DENTAL MATERIALS XXX (2017) XXX–XXX

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Response to antiseptic agents of periodontal pathogens in *in vitro* biofilms on titanium and zirconium surfaces

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ARTICLE INFO

Article history:
Received 10 October 2016
Received in revised form
19 January 2017
Accepted 31 January 2017
Available online xxx

Keywords:
Peri-implant biofilm
Aggregatibacter actinomycetemcomitans
Porphyromonas gingivalis
Fusobacterium nucleatum
Zirconium
Titanium
qPCR
Chlorhexidine
Essential oils
Cetyl-pyridinium-chloride

ABSTRACT

Objective. The aim of this study was to develop *in vitro* biofilms on SLA titanium (Ti-SLA) and zirconium oxide (ZrO_2) surfaces and to evaluate the effect of antiseptic agents on the number of putative periodontal pathogenic species.

Methods. An *in vitro* biofilm model was developed on sterile discs of Ti-SLA and ZrO_2. Three antiseptic agents [chlorhexidine and cetyl-pyridinium-chloride (CHX/CPC), essential oils (EEOOs) and cetyl-peridinium-chloride (CPC)] were applied to 72-h biofilms, immersing discs during 1 min in the antiseptic solution, either with or without mechanical disruption. Viable bacteria [colony forming units (CFU/mL)] were measured by quantitative polymerase chain reaction (qPCR) combined with propidium monoazide. A generalized lineal model was constructed to determine the effect of the agents on the viable bacterial counts of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and *Fusobacterium nucleatum* on each surface.

Results. The exposure to each antiseptic solution resulted in a statistically significant reductions in the number of viable target species included in the *in vitro* multi-species biofilm, on both Ti-SLA and ZrO_2 ($p < 0.001$) which was of up to 2 orders for *A. actinomycetemcomitans*, for *P. gingivalis* 2 orders on Ti-SLA and up to 3 orders on ZrO_2, and, for *F. nucleatum* up to 4 orders. No significant differences were found in counts of the tested bacteria between *in vitro* biofilms formed on both Ti-SLA and ZrO_2, after topically exposure to the antimicrobial agents whether the application was purely chemical or combined with mechanical disruption.

Significance. *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum* responded similarly to their exposure to antiseptics when grown in multispecies biofilms on titanium and zirconium surfaces, in spite of the described structural differences between these bacterial communities.

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http://dx.doi.org/10.1016/j.dental.2017.01.013
0109-5641/

1. Introduction

Biofilms are complex microbial communities developed on solid surfaces exposed to a wet environment [1]. In the oral cavity, different biofilms may be encountered attached to different solid oral surfaces, including teeth, prosthetic devices and dental implants [1–4]. The formation and maturation of biofilms on dental implant surfaces have been associated with the etiology of peri-implant mucositis and peri-implantitis, in a similar manner as the subgingival biofilm is associated with gingivitis and periodontitis [1–5].

Despite the similarities between biofilms on tooth and implant surfaces, some specific features might be attributed to the specific implant surface characteristics [4]. Previous *in vivo* and *in vitro* investigations have reported that surface characteristics such as roughness, surface free energy, wettability and degree of sterilization may affect biofilm formation and its bacterial three-dimensional distribution, although there is still controversy on the relevance of these differences. Recent studies evaluating biofilms on abutments, with different surface composition and topography, have shown that there is a correlation between surface roughness and viable biomass within the biofilm [6,7]. There is, however, controversy on which are the key factors guiding biofilm formation on implant surfaces, since in some studies using *in vitro* biofilm models, surface roughness seems to be the main factor [8–10], while in others, surface free energy, rather than roughness, seems to be the key factor determining initial bacterial adhesion [11,12]. Similarly, a positive correlation between surface roughness and bacterial colonization has been found in some models [13–15], while in others, certain titanium topographies seemed to inhibit bacteria adhesion together with the promotion of bone tissue formation [16,17]. Also, titanium purity, and not only surface topography, may influence early bacterial colonization [18]. Our research group, using an *in vitro* multibacterial species biofilm, has reported significant differences in biofilm thickness and three-dimensional structure, when comparing titanium and zirconium surfaces, with a higher number of initial and early colonizers (*Streptococcus oralis*, *Actinomyces naeslundii* and *Veillonella parvula*) on zirconium than on titanium surfaces [19]. These results are coincident with recent studies by de Avila et al. [20], reporting quantitative and qualitative differences between biofilms formed on titanium *versus* zirconium surfaces.

Antimicrobial agents, such as chlorhexidine (CHX), essential oils (EEOOs) or cetyl-pyridinium chloride (CPC), combined with mechanical debridement, are the gold standard therapy in the treatment of peri-implant mucositis and in the secondary prevention of peri-implantitis [21]. However, there is controversy whether implant micro-surface topography and chemistry influence the antimicrobial effect of these antimicrobial agents. This *in vitro* study was, therefore, aiming to assess whether the number of specific bacterial pathogens growing on *in vitro* biofilms over SLA titanium and zirconium oxide surfaces, were differentially affected when exposed to different antiseptic agents (alcohol-free EEOOSs, CPC and CHX/CPC).

2. Material and methods

2.1. Bacterial strains and culture conditions

Standard reference strains of *S. oralis* CECT 907T, *V. parvula* NCTC 11810, *A. naeslundii* ATCC 19039, *Fusobacterium nucleatum* DMSZ 20482, *Aggregatibacter actinomycetemcomitans* DSMZ 8324 and *Porphyromonas gingivalis* ATCC 33277 were used. These bacteria were grown on blood agar plates (Blood Agar Oxoid No 2; Oxoid, Basingstoke, UK), supplemented with 5% (v/v) sterile horse blood (Oxoid), 5.0 mg L^{-1} hemin (Sigma, St. Louis, MO, USA) and 1.0 mg L^{-1} menadione (Merck, Darmstadt, Germany) in anaerobic conditions (10% H_2, 10% CO_2, and balance N_2) at 37 °C for 24–72 h.

2.2. Material specimens

Sterile discs of 5 mm of diameter made of two different surface materials were used: (1) titanium with a SLA grade 2 surface (Ti-SLA) (Sand-blasted, Large grit, Acid-etched; Straumann; Institut Straumann AG, Basel, Switzerland), and (2) sterile zirconium oxide (ZrO_2), with a rough micro surface obtained after chemical treatment with a hot solution of hydrofluoric acid, according to a proprietary process of Institut Straumann AG (Institut Straumann AG, Basel, Switzerland). The resulting rough surface topography of ZrO_2 discs has a S_a value of 0.55 mm (standard deviation, SD = 0.01) with a rough surface topography similar the Ti-SLA surface implants when evaluated with scanning electron microscopy (SEM), although zirconium surfaces seemed to have a flatter profile with less porosity [S_a value of Ti-SLA surface of 1.17 mm (SD = 0.04)] [22].

2.3. Saliva preparation

Un-stimulated saliva was obtained from healthy volunteers in 10 mL aliquots at least 1.5 h after eating, drinking or tooth brushing. Each saliva sample was treated with 2.5 mmol L^{-1} DL–Dithiothreitol (Sigma) for 10 min with continuous stirring in order to reduce salivary protein aggregation. It was then centrifuged (10 min, 4 °C and 12,000 rpm) and the obtained supernatant was diluted (1:1) with phosphate buffered saline (PBS; pH = 7.4). The sample was then filtered and sterilized through a 0.22 μm pore size Millex GV low-protein-binding filter X50 (Millipore, Millipore Corporation Bedford, USA) and stored at −20 °C. The efficacy of this protocol was validated by plating processed saliva samples onto supplemented blood agar plates for 72 h at 37 °C, when confirmed by lack of any bacterial growth on either aerobically or anaerobically incubated plates.

2.4. Biofilm development

Biofilms were generated using the method described by Sánchez et al. [23] with slightly different bacterial concentrations when preparing the bacterial suspension. Briefly, planktonic cultures of each bacteria were grown anaerobically in a protein-rich medium containing brain–heart infusion (BHI) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) supplemented with 2.5 g L^{-1} mucin (Oxoid), 1.0 g L^{-1}

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yeast extract (Oxoid), 0.1 g L^{-1} cysteine (Sigma), 2.0 g L^{-1} sodium bicarbonate (Merck), 5.0 mg L^{-1} hemin (Sigma), 1.0 mg L^{-1} menadione (Merck) and 0.25% (v/v) glutamic acid (Sigma). Upon mid-exponential phase, the cells were mixed in modified BHI medium containing 10^3 colony forming units (CFU) mL^{-1} for *S. oralis*, 10^5 CFU mL^{-1} for *V. parvula* and *A. naeslundii*, and 10^8 CFU mL^{-1} for *F. nucleatum*, *A. actinomycetemcomitans* and *P. gingivalis*. Ti-SLA and ZrO_2 sterile discs were coated with treated saliva for 4 h at 37 °C in sterile plastic tubes, and then placed in the wells of a 24-well tissue culture plate (Greiner Bio-one, Frickenhausen, Germany). Each well was inoculated with 1.5 mL mixed bacteria suspension prepared and incubated in anaerobic conditions (10% H_2, 10% CO_2, and balance N_2) at 37 °C for 72 h. Plates containing only culture medium were also incubated to check for sterility.

2.5. SEM analysis

Before SEM analysis, three discs of each material (Ti-SLA and ZrO_2) covered with biofilms grown *in vitro* for 72 h were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde for 4 h at 4 °C. After fixation, the discs were washed twice in PBS and again twice in sterile water (immersion time per washed, 10 min) and dehydrated through a series of graded ethanol solutions (50, 60, 70, 80, 90 and 100%; immersion time per series, 10 min). Then, the samples were critical point dried, sputter-coated with gold and analyzed by electron microscopy JSM 6400 (JSM6400; JEOL, Tokyo, Japan), with back-scattered electron detector and an image resolution of 25 KV. Analyses were carried out at ICTS National Centre of Electronic Microscopy (Campus of International Excellence Moncloa, University Complutense, Madrid, Spain).

2.6. Exposure to antimicrobial compounds

The following commercially available antiseptic mouth rinse formulations were used: (1) 0.12% CHX and 0.05% CPC without alcohol (CHX/CPC) (Perio-Aid tratameiento®; Dentaid, Cerdanyola, Spain), (2) a combination of four EEOOs (thymol 0.06%, eucalyptol 0.09%, methyl salicylate 0.06% and menthol 0.01%) without alcohol (Listerine® Zero; Johnson & Johnson, Madrid, Spain), an (3) 0.05% CPC without alcohol (Oral B-ProExpert, Procter & Gamble, Weybridge, Surrey, UK). PBS was used as a negative control solution.

To monitor the bactericidal action of the three tested mouth rinses on 72 h biofilms, the discs were immersed during 1 min in the antiseptic solution and in PBS as control, with and without mechanical disruption by means of agitation through vortex at room temperature, which provided constant stirring at 90 rpm. After this exposure, the discs were sequentially rinsed in 2 mL of sterile PBS (immersion time per rinse, 10 s), three times, to remove the antiseptic solutions.

In each experiment, the three antimicrobial agents and the control solutions were tested with and without mechanical disruption (agitation). These experiments were repeated three times on different days using fresh bacterial cultures with trios of biofilms for each independent outcome variables.

2.7. Microbiological outcomes

After the antimicrobial treatment, biofilms were disrupted by vortex for 2 min in 1 mL of PBS. To discriminate between DNA from live and dead bacteria, propidium monoazide (PMA) (Biotium Inc., Hayword, CA, USA) was used. The use of this PMA dye has shown the ability to distinguish between viable and irreversibly damaged cells and hence when combined with quantitative polymerase chain reaction (qPCR) to detect the DNA from viable bacteria [24]. PMA was added to sample tubes containing 250 μL of disaggregated biofilm cells, at a final concentration of 100 μM. Following an incubation period of 10 min at 4 °C in the dark, the samples were subjected to light-exposure for 20 min, using a 550 W halogen light source, placed 20 cm above the samples. During this exposure, the sample tubes were laid horizontally on ice to avoid excessive heating. After PMA photo-induced DNA cross-linking, the cells were centrifuged at 12,000 rpm for 3 min prior to DNA isolation. To control for any possible influence of the experimental process on bacterial viability, the same procedure (incubation at 4 °C and exposure to light source) but without the exposure to PMA, was used as negative control.

Bacterial DNA was isolated from all biofilms using a commercial kit (MolYsis Complete5; Molzym GmbH & CoKG, Bremen, Germany), following manufacturer's instructions (the protocol for bacterial DNA extraction was followed from step 6, avoiding preliminary steps) and the hydrolysis 5′nuclease probe assay qPCR method was used for detecting and quantifying the bacterial DNA. The qPCR amplification was performed following a protocol previously optimized by our research group, using primers and probes targeted against 16S *rRNA* gene [obtained through Life Technologies Invitrogen (Carlsbad, CA, USA) and Applied Biosystems (Carlsbad, CA, USA) [19].

Each DNA sample was analyzed in duplicate. Quantification cycle (Cq) values, previously known as cycle threshold (Ct) values, describing the PCR cycle number at which fluorescence rises above the baseline, were determined using the provided software package (LC 480 Software 1.5; Roche Diagnostic GmbH; Mannheim, Germany). Quantification of viable cells by qPCR was based on standard curves [19]. The correlation between Cq values and CFU/mL was automatically generated through the software (LC 480 Software 1.5; Roche).

All assays were developed with a linear quantitative detection range established by the slope range of 3.3–3.6 cycles/log decade, $r^2 > 0.997$ and an efficiency range of 1.9–2.0.

Measures to avoid carryover DNA were established. In spite of this, when non-template control (NTC) was detectable, the limit of detection was established on the last value of the standard curve that holds five cycles of difference with NTC.

2.8. Statistical analyses

The following independent variables were considered: (1) the material surface (Ti-SLA and ZrO_2), (2) the mechanical disruption applied by constant agitation (with or without), (3) type of antiseptic/control (PBS, CHX/CPC, EEOOs or CPC), and (4) their interaction. The number of viable bacteria present on *in vitro* biofilms formed on SLA titanium and zirconium oxide surfaces and measured as viable CFU/mL of *A. actinomycetemcomitans*, *P.*

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gingivalis and *F. nucleatum* was the primary dependent outcome variable.

An experiment-level analysis was performed for each study parameter (n = 9 or 72 for qPCR results). Shapiro–Wilk goodness-of-fit tests and distribution of data were used to assess normality. Data were expressed as means and 95% confidence intervals (CI).

In order to compare the effect of material surface, type of antiseptic with or without mechanical disruption, and their interaction on the main outcome variable (CFU/ml) a general lineal model was constructed for each bacterium (*A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum*) using the method of maximum likelihood and Bonferroni corrections for multiple comparisons.

Results were considered statistically significant at $p < 0.05$. A software package (IBM SPSS Statistics 21.0; IBM Corporation, Armonk, NY, USA) was used for all data analysis.

3. Results

SEM observations revealed notable differences in the structure of biofilms depending on the surface topography (ZrO_2 *versus* Ti-SLA) after 72 h of incubation (Fig. 1). Although in both surfaces the bacterial species *F. nucleatum* seemed to play a key structural role on Ti-SLA discs the bacterial cells formed a compact consortium, depicting a crater-like architecture (Fig. 1A, C & E). Conversely, on the ZrO_2 discs, the biofilms had a complex morphology, in which *F. nucleatum* formed a

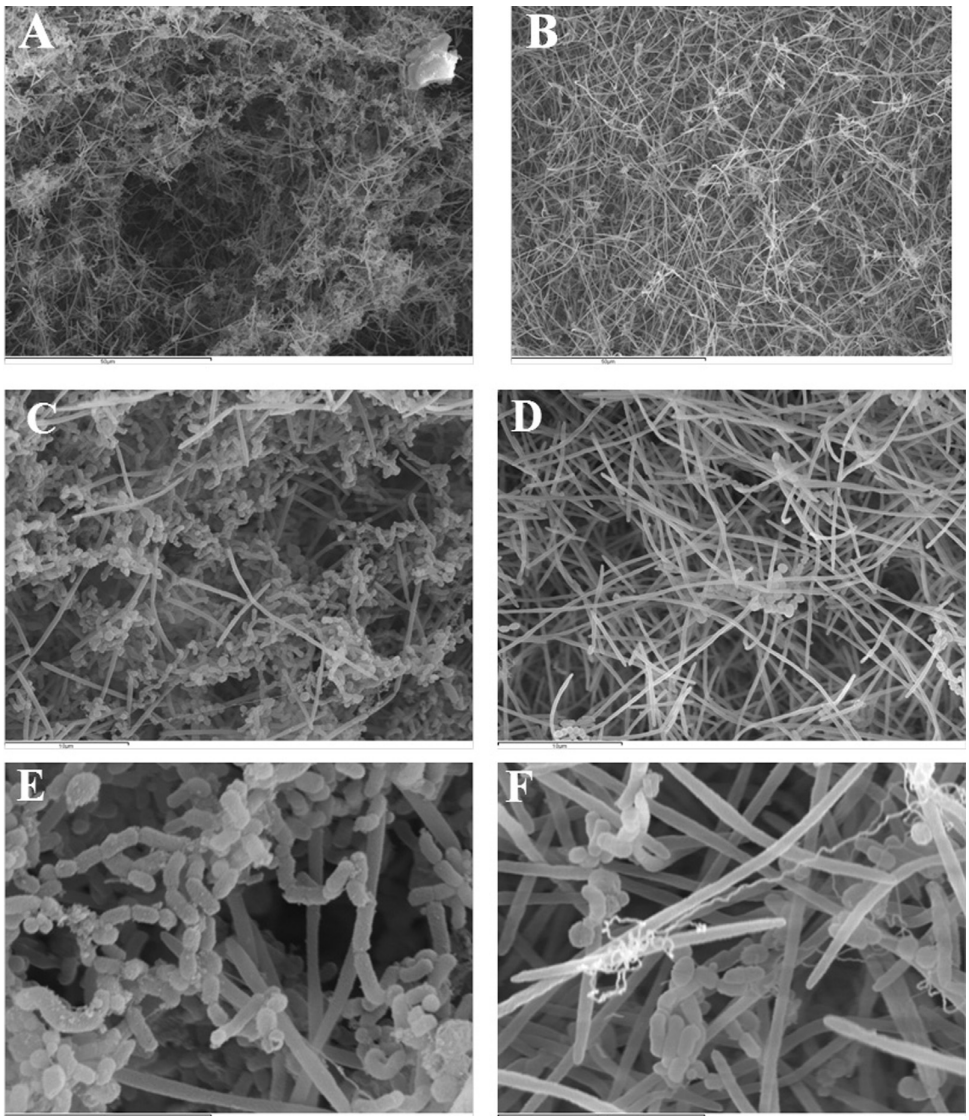

Fig. 1 – Scanning electron microscopy (SEM) of oral biofilms formed *in vitro* on Ti-SLA (A, C, E) and ZrO_2 (B, D, F) discs. The biofilms formed after 72 h was composed of *Streptococcus oralis*, *Veillonella parvula*, *Actinomyces naeslundii*, *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*. It can be observed the different bacterial disposition as well as the different three-dimensional structure of the biofilms promoted by both implant surfaces. Magnification: (A, D): 3000×; (B, E): 5000×; (C, F): 10,000×.

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Table 1 – Independent effect of each antimicrobial agent on the mean number of viable bacteria in the *in vitro* multi-species biofilm (CFU/mL). Data were expressed as means and 95% confidence intervals (95% CI). PBS: phosphate buffer saline; CHX/CPC: chlorhexidine and cetyl-pyridinium-chloride; EEOOs: essential oils; CPC: cetyl-pyridinium-chloride.

	Viable CFU/mL [mean (95% CI)]			
	Control biofilm	Treatment with the corresponding antimicrobial agent		
	(PBS)	EEOOs	CPC	CHX/CPC
A. actinomycetemcomitans	3.6×10^6 (2.4×10^6; 4.8×10^6)	2.2×10^5 (-9.9×10^5; 1.4×10^6)	2.0×10^5 (-1.0×10^6; 1.4×10^6)	1.5×10^5 (-1.1×10^6; 1.4×10^6)
P. gingivalis	5.7×10^7 (4.8×10^7; 6.5×10^7)	5.3×10^6 (-3.5×10^6; 1.4×10^7)	5.9×10^6 (-2.9×10^6; 1.5×10^7)	2.3×10^6 (-6.5×10^6; 1.1×10^7)
F. nucleatum	8.3×10^6 (5.4×10^6; 1.1×10^7)	8.3×10^4 (-2.8×10^6; 3.0×10^6)	4.0×10^5 (-2.5×10^6; 3.3×10^6)	2.2×10^4 (-2.9×10^6; 2.9×10^6)

core, while the other micro-colonies adhered forming a thick network (Fig. 1B, D & F).

Despite the different structural arrangement observed, the analysis by qPCR of biofilms on both surfaces indicates no differences in the number of viable periodontal pathogens measured (viable CFU/mL). Depending on the implant material, Ti-SLA and ZrO_2 surfaces, the mean numbers of viable *A. actinomycetemcomitans* grown in the *in vitro* biofilms were 1.1×10^6 (95% CI: 2.6×10^5; 2.0×10^6) CFU/mL on Ti-SLA surface and 9.9×10^5 (95% CI: 1.3×10^5; 1.8×10^6) on ZrO_2. Corresponding data for *P. gingivalis* were, respectively, 1.5×10^7 (95% CI: 8.5×10^6; 2.1×10^7) and 2.0×10^7 (95% CI: 1.4×10^7; 2.7×10^7) CFU/mL, and for *F. nucleatum* 2.1×10^6 (95% CI: 8.1×10^4, 4.2×10^6) CFU/mL on Ti-SLA and 2.3×10^6 (95% CI: 2.3×10^5; 4.3×10^6) on ZrO_2. Differences between the two materials were not statistically significant for any of the targeted bacterial species [*A. actinomycetemcomitans* ($p = 0.832$), *P. gingivalis* ($p = 0.200$) and *F. nucleatum* ($p = 0.921$)] ($n = 72$ for each bacterium).

The independent effect of each antimicrobial agent compared to the negative control solution (PBS) on the mean number of viable tested bacteria in biofilms is shown in Table 1. For *A. actinomycetemcomitans,* the viable bacteria suffered a reduction by more than 1 order of magnitude after exposure to the three antimicrobial solutions, dropped from 3.6×10^6 to 1.5×10^5 CFU/mL when biofilms were treated with the most effective antiseptic solution (CHX/CPC). The same occurred with *P. gingivalis,* reducing the counts from 5.7×10^7 to 2.3×10^6 CFU/mL. *F. nucleatum* decreased up to 2 orders of magnitude, from 8.3×10^6 to 2.2×10^4 CFU/mL. The reductions were statistically significant for all targeted bacteria and for each antiseptic solution ($p < 0.01$). However, no significant differences were observed when comparing the effects among each antimicrobial for any of the tested bacteria ($p = 1.000$; $n = 72$ in each case).

Regarding the independent effect of mechanical disruption in the number of viable bacteria on the biofilm, it can be observed that *A. actinomycetemcomitans* reduced the number of viable cells from 2.0×10^6 (95% CI: 1.2×10^6; 2.9×10^6) to 6.0×10^4 (95% CI: -7.9×10^5; 9.1×10^5) CFU/mL, *P. gingivalis* from 3.4×10^7 (95% CI: 2.8×10^7; 4.0×10^7) to 8.3×10^5 (95% CI: -5.4×10^6; 7.0×10^6) CFU/mL, and *F. nucleatum* from 4.4×10^6 (95% CI: 2.3×10^6; 6.4×10^6) to 4.9×10^4 (95% CI: -2.0×10^6; 2.1×10^6) CFU/mL. These reductions were statistically significant for all targeted bacteria ($p < 0.01$; $n = 72$ in each case).

The combined effect of type of antiseptic and mechanical disruption over material surface (Ti-SLA or ZrO_2), on the mean number of viable CFU/mL is presented in Tables 2 and 3. For biofilms formed on Ti-SLA, *A. actinomycetemcomitans* reduced significantly their mean viable number from 7.2×10^6 to 3.4×10^5 CFU/mL when biofilms were treated with the most effective antiseptic solution (CHX/CPC). This reduction increased up to 2.0×10^4 CFU/mL when the antiseptic immersion was combined with mechanical disruption (Table 2). In a similar manner the viable number of *P. gingivalis* reduced significantly when the biofilm grown on Ti-SLA were exposed to CHX/CPC, from 8.7×10^7 to 3.2×10^6 CFU/mL, reaching 1.5×10^5 viable CFU/mL when were mechanically disrupted by agitation. *F. nucleatum* also significantly reduced its mean viable numbers, but in this case the highest effect was obtained with CPC dropping from 1.6×10^7 up to 9.8×10^3 CFU/mL under dynamic condition (Table 2). For the three target bacterial species, a statistically significant reduction was found when comparing the mean number of viable target bacteria in control biofilms (exposed to PBS) with biofilms exposed to the three antimicrobial agents (CHX/CPC, EEOOs or CPC), with or without mechanical disruption ($p < 0.001$). In any case, no statistically significant differences were found in the mean reductions of target bacteria when comparing the effect of the three antiseptics among them ($p = 1.000$), even though, in the Ti-SLA surface, the highest magnitude of reduction for *A. actinomycetemcomitans* and *P. gingivalis* was obtained with the use of CHX/CPC, while for *F. nucleatum* the biggest effect was observed with CPC.

For the ZrO_2 surface the number of viable *A. actinomycetemcomitans* in the biofilm decreased from 7.2×10^6 to 2.1×10^5 CFU/mL when treated with CHX/CPC, up to 1.9×10^4 subject to mechanical disruption (Table 2). *P. gingivalis* reduced the number of viable cells from 1.4×10^8 to 5.7×10^6 CFU/mL, reaching 1.2×10^5 when mechanical disruption was combined with CHX/CPC as the most effective antimicrobial (Table 2). *F. nucleatum* suffered a significant reduction in their vitality from 1.7×10^7 to 2.4×10^4 CFU/mL when biofilms were treated with CHX/CPC, that decreased to 6.0×10^3 when mechanical disruption was also applied (Table 2). For the three target bacteria, when compared the number of viable bacteria of control biofilms (exposed to PBS) with biofilms exposed to the three antimicrobial agents (CHX/CPC, EEOOs or CPC), with or without mechanical disruption, it could be observed a significant reduction of vitality ($p < 0.001$ in all cases). The anti-

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Table 2 – Effect of the type of material, antiseptic and agitation on number of viable *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and *Fusobacterium nucleatum* expressed as mean values of viable colony forming units per biofilm (CFU/mL) in a multi-species biofilm generated on SLA titanium and zirconium oxide surfaces. All samples were treated with Propidium Monoazide before DNA extraction. PBS: phosphate buffer saline; CHX/CPC: chlorhexidine and cetyl-pyridinium-chloride; EEOOs: essential oils; CPC: cetyl-pyridinium-chloride.

Material	Treatment	Agitation	Viable CFU/mL per biofilm [means (95% CI)]		
			A. actinomycetemcomitans	*P. gingivalis*	*F. nucleatum*
Titanium (Ti-SLA)	PBS	No	7.2×10^6 (4.8×10^6; 9.6×10^6)	8.7×10^7 (7.0×10^7; 1.0×10^8)	1.6×10^7 (1.0×10^7; 2.2×10^7)
	EEOOs		4.4×10^5 (-2.0×10^6; 2.9×10^6)	8.9×10^6 (-8.6×10^6; 2.6×10^7)	1.4×10^5 (-5.7×10^6; 5.9×10^6)
	CPC		5.6×10^5 (-1.9×10^6; 9.3×10^6)	1.3×10^7 (-4.4×10^6; 3.1×10^7)	5.7×10^5 (-5.2×10^6; 6.4×10^6)
	CHX/CPC		3.4×10^5 (-2.1×10^6; 2.8×10^6)	3.2×10^6 (-1.4×10^7; 2.1×10^7)	3.9×10^4 (-5.8×10^6; 5.8×10^6)
	PBS	Yes	1.6×10^5 (-2.3×10^6; 2.6×10^6)	3.1×10^6 (-1.4×10^7; 2.1×10^7)	2.0×10^5 (-5.6×10^6; 6.0×10^6)
	EEOOs		1.4×10^5 (-2.3×10^6; 2.6×10^6)	1.0×10^6 (-1.6×10^7; 1.9×10^7)	3.9×10^4 (-5.8×10^6; 5.8×10^6)
	CPC		4.7×10^4 (-2.4×10^6; 2.5×10^6)	5.9×10^5 (-1.7×10^7; 1.8×10^7)	9.8×10^3 (-5.8×10^6; 5.8×10^6)
	CHX/CPC		2.0×10^4 (-2.3×10^6; 2.4×10^6)	1.5×10^5 (-1.7×10^7; 1.8×10^7)	1.8×10^4 (-5.8×10^6; 5.8×10^6)
Zirconium (ZrO2)	PBS	No	7.2×10^6 (-4.8×10^6; 9.6×10^6)	1.4×10^8 (1.2×10^8; 1.5×10^8)	1.7×10^7 (1.1×10^7; 2.3×10^7)
	EEOOs		2.5×10^5 (-2.2×10^6; 2.7×10^6)	1.1×10^7 (-6.8×10^6; 2.8×10^7)	1.3×10^5 (-5.7×10^6; 5.9×10^6)
	CPC		1.7×10^5 (-2.2×10^6; 2.6×10^6)	9.6×10^6 (-7.9×10^6; 2.7×10^7)	1.0×10^6 (-4.8×10^6; 6.8×10^6)
	CHX/CPC		2.1×10^5 (-2.2×10^6; 2.6×10^6)	5.7×10^6 (-1.2×10^7; 2.3×10^7)	2.4×10^4 (-5.8×10^6; 5.8×10^6)
	PBS	Yes	4.2×10^4 (-2.4×10^6; 2.6×10^6)	1.1×10^6 (-1.6×10^7; 1.9×10^7)	8.9×10^4 (-5.7×10^6; 5.9×10^6)
	EEOOs		3.3×10^4 (-2.4×10^6; 2.4×10^6)	3.1×10^5 (-1.7×10^7; 1.8×10^7)	2.5×10^4 (-5.8×10^6; 5.8×10^6)
	CPC		2.2×10^4 (-2.3×10^6; 2.4×10^6)	1.7×10^5 (-1.7×10^7; 1.8×10^7)	6.3×10^3 (-5.8×10^6; 5.8×10^6)
	CHX/CPC		1.9×10^4 (-2.4×10^6; 2.4×10^6)	1.2×10^5 (-1.7×10^7; 1.8×10^7)	6.0×10^3 (-5.8×10^6; 5.8×10^6)

septic CHX/CPC was the antimicrobial agent demonstrating higher reductions in the number of all viable target pathogens (Table 2), both with and without mechanical disruption, for the three tested bacteria. However, no significant differences were observed when comparing the effects of each antimicrobial for any of the targeted bacteria ($p = 1.000$ in all cases).

Table 3 shows there were not any statistically significant differences in the mean number of viable *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum*, respectively, in response to the corresponding solution (EEOOs, CPC or CHX/CPC) between *in vitro* biofilms on Ti-SLA and ZrO2 surfaces, either under constant agitation at 90 rpm or without agitation (Table 3).

4. Discussion

The results from this investigation, on *in vitro* multi-bacterial species biofilms formed over Ti-SLA and ZrO_2 surfaces, have shown that *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum* suffer a similar decrease in their viability (viable CFU/mL) when topically exposed to antimicrobial agents (CHX/CPC, EEOOs and CPC), whether the application was purely chemical or combined with mechanical disruption by agitation. Also, the results, in agreement with those proposed by other authors, reported that although early bacterial colonization may be influenced by different implant surface characteristics [6,7,11,12,14,18], the mature biofilm is quite similar over the two implant surfaces, in terms of the number of bacteria [9,10,19,20,25,26]. This was confirmed by the presence of similar number of viable bacteria on SLA-Ti and ZrO_2surfaces despite differences in density, thickness or three-dimensional structures of these anaerobic communities.

Scientific evidence on the effectiveness of antimicrobial agents, on biofilms formed on implant surfaces, is still limited, and even scarcer in observing the possible difference in the response of periodontal pathogens included in microbial communities formed on different implant surfaces. This study has reported that all tested antimicrobial agents (CHX/CPC, EEOOs and CPC) significantly reduced bacterial viability ($p < 0.05$) of each target periodontal pathogenic bacteria, being CHX/CPC the most effective one, although no significant differences were detected among agents. These results are in concordance with the available literature reporting *in vitro* studies. Al-Radha et al. [27] evaluated the *in vitro* effects of natural antimicrobial agents and CHX gluconate in the reduction of biofilm development on titanium and zirconium dental implant surfaces using a constant depth film fermenter (CDFF), finding a significant decrease in bacterial adhesion in the first 2 days after antimicrobial treatments in both surfaces. Also, the efficacy of 0.2% CHX, EEOOs, stannous fluoride and hexetidine associated with methylparaben and propylparaben, in reducing *in vitro* peri-implant biofilm, was compared by Baffone et al. [28], concluding that CHX was the most effective agent. Similarly, Erriu et al. [29] reported an antimicrobial effect on *A. actinomycetemcomitans* when treated with mouth rinses based on free-alcohol EEOOs and 0.12% CHX, being CHX the most effective agent. Recently, Verardi et al. [30] have studied the potential effects of antiseptics such as CHX, chloramine T (CHT), triclosan and EEOOs on bacterial adhesion and on biofilm formation using a microcosm technique, in which the oral environment and periodontal conditions were simulated *in vitro* on titanium discs with different surface treatments (smooth surface, acid-etched smooth surface, sand-blasted surface and sand-blasted and acid-etched). They observed that the different antiseptics reduced the amount of bacteria over titanium implants and no significant differences were observed between CHX and CHT or EEOOs. The available evidence also agrees in considering CHX as the most effective agent in *in vivo* plaque reduction [31–37]

Please cite this article in press as: Sánchez MC, et al. Response to antiseptic agents of periodontal pathogens in *in vitro* biofilms on titanium and zirconium surfaces. Dent Mater (2017), http://dx.doi.org/10.1016/j.dental.2017.01.013

Table 3 – Effect of type of material, antiseptic and agitation on number of viable *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and *Fusobacterium nucleatum* in a multi-species biofilm when comparing SLA titanium (Ti-SLA) and zirconium oxide (ZrO_2) surfaces. PBS: phosphate buffer saline; EEOOs: essential oils; CPC: cetyl-pyridinium-chloride; CHX/CPC: chlorhexidine and cetyl-pyridinium-chloride.

Material	Chemical treatment	Agitation	Mean differences									
			A. actinomycetemcomitans				*P. gingivalis*				*F. nucleatum*			
			Mean	Confidence interval		p Value	Mean	Confidence interval		p Value	Mean	Confidence interval		p Value
				Lower	Upper			Lower	Upper			Lower	Upper	
Ti-SLA/ZrO_2	PBS	No	6.4×10^4	-3.3×10^6	3.5×10^6	0.970	-4.8×10^7	-7.3×10^7	-2.3×10^7	0.000	-8.9×10^5	-9.1×10^6	7.3×10^6	0.829
	EEOOs		1.9×10^5	-3.2×10^6	3.6×10^6	0.912	-1.8×10^6	-2.7×10^7	2.3×10^7	0.884	1.0×10^4	-8.2×10^6	8.2×10^6	0.998
	CPC		4.0×10^5	-3.0×10^6	3.8×10^6	0.819	3.5×10^6	-2.1×10^7	2.8×10^7	0.778	-4.4×10^5	-8.6×10^6	7.8×10^6	0.916
	CHX/CPC		1.4×10^5	-3.3×10^6	3.5×10^6	0.937	-2.5×10^6	-2.7×10^7	2.2×10^7	0.843	1.5×10^4	-8.2×10^6	8.2×10^6	0.997
Ti-SLA/ZrO_2	PBS	Yes	1.2×10^5	-3.3×10^6	3.5×10^6	0.945	2.0×10^6	-2.3×10^7	2.7×10^7	0.872	1.1×10^5	-8.1×10^6	8.3×10^6	0.979
	EEOOs		1.0×10^5	-3.3×10^6	3.5×10^6	0.952	7.1×10^5	-2.4×10^7	2.5×10^7	0.955	1.4×10^4	-8.2×10^6	8.2×10^6	0.997
	CPC		2.5×10^4	-3.4×10^6	3.4×10^6	0.988	4.1×10^5	-2.4×10^7	2.5×10^7	0.974	3.4×10^3	-8.2×10^6	8.2×10^6	0.999
	CHX/CPC		1.3×10^3	-3.4×10^6	3.4×10^6	0.999	3.0×10^4	-2.5×10^7	2.5×10^7	0.998	1.2×10^4	-8.2×10^6	8.2×10^6	0.998

This study has also found that there were not any statistically significant differences in the mean number of viable *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum*, in response to the different mouth rinses (EEOOs, CPC or CHX/CPC) between *in vitro* biofilms on Ti-SLA and ZrO_2 surfaces, either with or without mechanical disruption. While some surface parameters might significantly affect biofilm formation and bacterial three-dimensional distribution within implant-associated biofilms, there is still controversy whether the implant micro-surface topography and chemistry may influence the ability of antimicrobial agents to directly influence biofilm vitality. Further research will be necessary to elucidate this matter.

5. Conclusions

Within the limitations of this *in vitro* study, it can be concluded that *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum* suffer a similar decrease in their mean number of viable cell (CFU/mL) when they are included on multi-bacterial species biofilms formed on either SLA-Ti and ZrO_2 surfaces, after being exposed to antimicrobial agents (CHX/CPC, EEOOs and CPC), whether the application was purely chemical or combined with mechanical disruption (with agitation).

Acknowledgements

This study was funded by CDTI (Center for the Development of Industrial Technology) of the Spanish Ministry of Science and Innovation and the company DENTAID (Barcelona, Spain), contract number 332/2011. Special thanks to Dr. Rubén León and Dr. Vanessa Blanc for their collaboration. We also thank Institut Straumann AG (Basel, Switzerland) for providing the implant materials and to Dr. Ana María Vicente for her support in the technical microscopy services at the ICTS National Centre of Electron Microscopy, University Complutense, Madrid, Spain.

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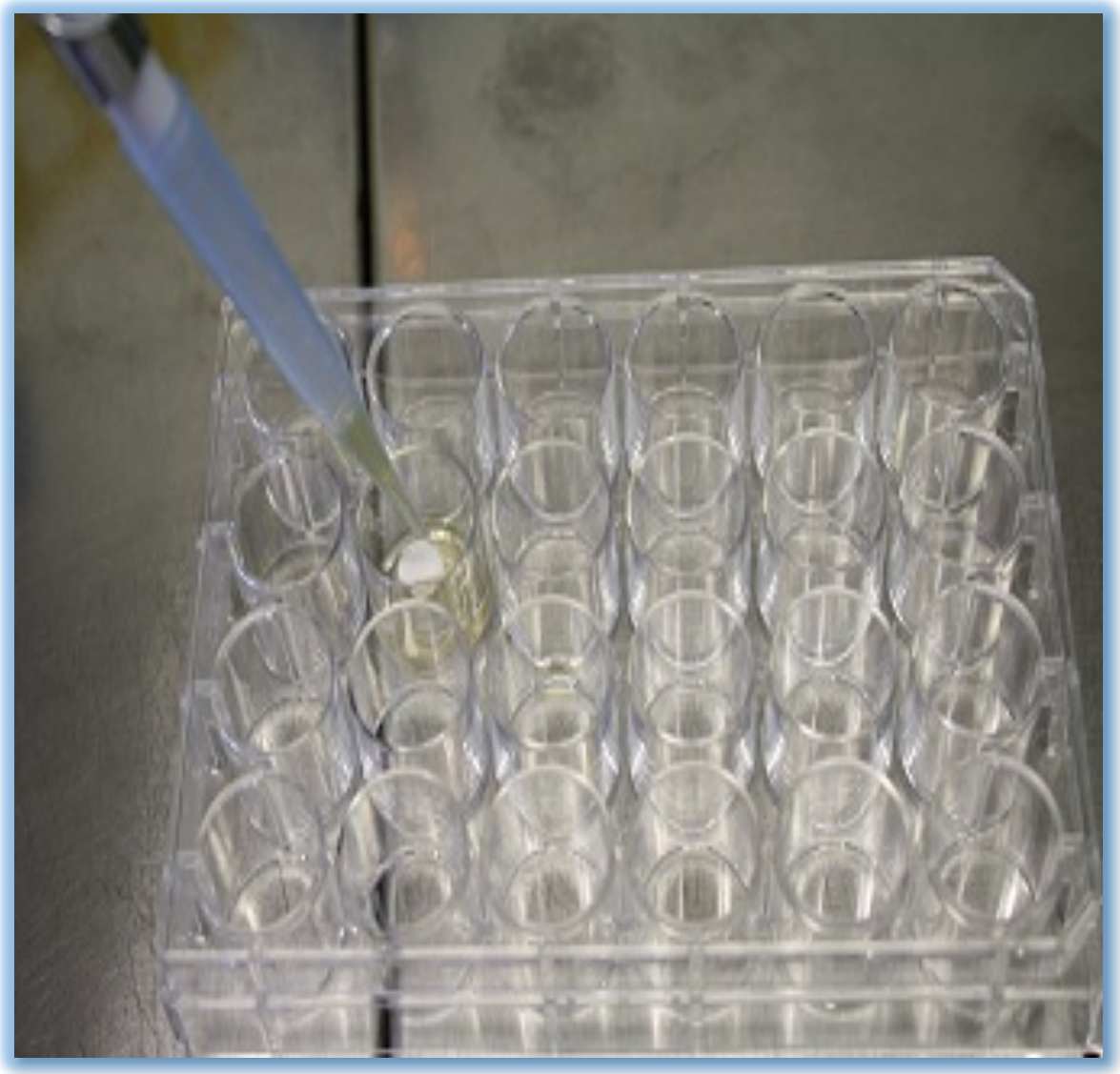
Please cite this article in press as: Sánchez MC, et al. Response to antiseptic agents of periodontal pathogens in *in vitro* biofilms on titanium and zirconium surfaces. Dent Mater (2017), http://dx.doi.org/10.1016/j.dental.2017.01.013

Discusión



Los resultados obtenidos en esta serie de estudios han demostrado que el modelo de biofilm *in vitro* empleado es válido, no sólo como herramienta, para desarrollar biofilms periimplantarios *in vitro*, si no también para comparar el efecto antibacteriano de distintos agentes, ya sea sobre superficies de implantes o de dientes. El modelo de biofilm *in vitro* seleccionado ha sido validado en estudios previos (Sánchez et al. 2011; Blanc et al. 2014; Sánchez et al. 2014), siendo relativamente fácil de cultivar y asegurando el desarrollo de un biofilm subgingival dental o periimplantario para una simulación realista de las condiciones orales, al haber inoculado seis bacterias que incluyen colonizadores iniciales como el *S. oralis*, intermedios, pertenecientes a los géneros *Actinomyces* o *Veillonella* y colonizadores tardíos incluyendo a *A. actinomycetencomitans* y *P. gingivalis*.

1 Comparación de la estructura y cinética bacteriana en un modelo de biofilm in vitro desarrollado en tres superficies diferentes: hidroxiapatita, titanio y zirconio.

El análisis estructural muestra que los biofilms se desarrollaron en los tres materiales testados. Sin embargo, las distintas superficies demostraron diferencias en la estructura tridimensional del biofilm, incluso en etapas tempranas, y esas diferencias se mantuvieron a lo largo del tiempo. La estructura, estudiada a través de CLSM y LTSM, así como su cinética y el número de las diferentes especies bacterianas, analizadas a través de qPCR, fue diferente, de manera estadísticamente significativa, al comparar la hidroxiapatita con las superficies de titanio y zirconio, mostrando biofilms más finos con un mayor número de bacterias al alcanzar el estado maduro (72 h.). Los biofilms en la superficie de zirconio fueron significativamente más finos que en hidroxiapatita y titanio.

A través de LTSM, la estructura tridimensional mostró diferencias tanto en la deposición del exopolisacárido (EPS) como en la organización de las células bacterianas. En la superficie de titanio, se observó una clara identificación de las "columnas" bacterianas y los canales de circulación, mientras que en las superficies de zirconio, el biofilm adoptaba una morfología de tela de araña.

En la literatura se encuentran estudios que emplearon SEM y muestran resultados similares al comparar zirconio y titanio *in vivo* en 24 h. Estos estudios reportan que el porcentaje de área cubierta por el biofilm en zirconio fue significativamente menor que en superficies de titanio (Scarano et al. 2004). Schmidlin y colaboradores (Schmidlin et al. 2013) también emplearon SEM, y sin embargo describieron una estructura de biofilm similar al comparar diente y superficies de titanio.

Con el empleo de la qPCR se ha observado que la formación de biofilm y su dinámica es similar en las tres superficies testadas. Este biofilm de crecimiento dinámico coincide con las descripciones de la formación de biofilm en superficies dentales (Lee y Wang 2010). Una hora después de la inoculación bacteriana, las seis especies se detectaron dentro de los biofilms en las superficies de titanio, lo cual es coincidente con la cinética bacteriana descrita de 30 a 60 minutos después de colocar el implante (Quirynen et al. 2006; Furst et al. 2007; Nascimento et al. 2013). Los biofilms testados continuaron su crecimiento hasta llegar a una meseta con un pico de madurez a las 72 h. en las tres superficies analizadas, a pesar de que el número de bacterias fue significativamente más alto en las superficies de hidroxiapatita. Este dato también es coherente con los estudios que comparan la carga de bacterias totales entre dientes y muestras de implantes (Furst et al. 2007). Al contrario, no se encontraron diferencias estadísticamente significativas en el número de bacterias al comparar superficies de

titanio y zirconio, sugiriendo que ambas superficies son igualmente susceptibles a la acumulación de placa bacteriana.

Sin embargo, hay conflicto en los resultados encontrados en la literatura sobre los posibles efectos de la superficie del implante en la formación y maduración de la placa. Rimondini et al. (2002) estudiaron *in vitro* la adhesión bacteriana al titanio y a dos tipos de superficies de zirconio y señalaron que el zirconio mostraba significativamente mayor adherencia a *S. mutans* que el titanio después de 24 h, mientras que *S. sanguis* parecía adherirse más fácilmente al titanio. No se encontraron diferencias para *Actinomyces* spp. A la inversa, Lee et al. (2011) no encontraron diferencias estadísticamente significativas en la adhesión bacteriana *in vitro* (*S. sanguis*) entre titanio y zirconio después de 2 h de incubación. Al-Radha et al. (2012) observaron que *Streptococcus mitis* tenía menor afinidad para adherirse al zirconio que a las superficies de titanio después de 6 h de formación de biofilm *in vitro*. Schmidlin et al. (2013) demostraron en superficies de titanio cinéticas similares a lo que se describe en el presente estudio, con una adherencia inicial para *S. oralis*, *A. naeslundii*, *F. nucleatum* y *Veillonella* spp. a pesar de que hubo diferencias en los recuentos para cada bacteria (logaritmo de CFU/biofilm), debido, quizás, a las diferentes condiciones del modelo y métodos de evaluación. Con nuestro modelo, se ha observado que los colonizadores iniciales, *S. oralis*, *A. naeslundii* y *V. Parvula*, no difieren significativamente entre los biofilms de titanio y zirconio tras 1, 12 ó 24 h, lo cual está de acuerdo con Rimondini et al. (2002), el cual no encontró diferencias estadísticamente significativas en la adhesión bacteriana de *Actinomyces* spp. en las primeras 24 h. Todos estos estudios, sin embargo, son evaluaciones a corto plazo (24h o menos) y, por lo tanto, sólo estudian la adhesión bacteriana temprana.

Los resultados obtenidos en los biofilms maduros están también en consonancia con los descritos por de Oliveira et al. (2012) empleando qPCR, que no encontraron diferencias estadísticamente significativas entre el número de copias de ADN de *A. actinomycetencomitans*, *P. gingivalis* y bacterias totales, tanto para zirconio como para titanio, en superficies *in vivo*. De forma similar, Rimondini et al. (2002), no mostraron diferencias estadísticamente significativas en la colonización temprana *in vitro* de *P. gingivalis* (24 h.). Quirynen et al. (1994), sin embargo, postularon que los materiales con baja energía superficial, como el zirconio, acumulan más microorganismos cocoides y menos especies patógenas. Estos resultados se encuentran en consonancia con los resultados del presente estudio en lo referente al biofilm maduro en superficies de zirconio, donde la cantidad de *S. oralis* fue mayor que en titanio.

En conjunto, mientras la formación y dinámica de este modelo *in vitro* de biofilm fue similar independientemente de la superficie de inoculación (hidroxiapatita, titanio o zirconio), se han detectado diferencias estadísticamente significativas en lo que se refiere al grosor del biofilm y a la estructura tridimensional. Las implicaciones clínicas de este hallazgo deben ser evaluadas, pero se puede sugerir la hipótesis de que las diferencias descritas podrían tener un impacto en la susceptibilidad de los biofilms a los agentes antimicrobianos, los cuales podrían afectar a medidas preventivas (por ejemplo el uso de antisépticos como parte de los procedimientos de higiene oral) y enfoque de los tratamientos (como el uso de antimicrobianos locales o sistémicos). Además, las superficies más propensas a albergar biofilms complejos pueden ser más susceptibles para desarrollar enfermedades periimplantarias.

2. Comparación del efecto antibacteriano de diferentes dentífricos en un modelo de biofilm in vitro.

Los resultados de esta investigación han mostrado que el uso de los dentífricos en forma de "slurry" aplicado en un modelo de biofilm oral, que incluye los principales periodontopatógenos, fue capaz de detectar diferencias no sólo entre el control negativo y las pastas evaluadas, sino también entre agentes activos, en su efecto sobre los patógenos periodontales más relevantes (*P. gingivalis*, *A. actinomycetencomitans* y *F. nucleatum*). Los dos primeros están fuertemente asociados a la periodontitis **(**Consensus report 1996), mientras que el tercero juega un importante papel en el desarrollo del biofilm, construyendo la red que une los colonizadores tempranos y los patógenos orales reales **(**Kolenbrander 2000). Una sencilla exposición de 2 minutos del biofilm maduro a las pastas testadas, en continua agitación, para simular el cepillado *in vivo,* tiene como resultado un efecto bactericida, demostrando una reducción significativa en el recuento microbiano vivo al compararlo con el control. Este resultado se produjo no sólo al exponer el biofilm a los dos dentífricos con agentes antiplaca reconocidos (fluoruro de estaño y triclosán), sino también con dentífricos con fluoruros (1,45 y 2,5 de fluoruro de sodio), mostrando el efecto antibacteriano de estas pastas con fluoruro.

El principal objetivo de este estudio no era identificar un efecto inhibitorio de las pastas testadas, sino desarrollar un método efectivo para comparar el efecto antibacteriano de diferentes pastas sobre tres bacterias fuertemente asociadas con periodontitis. La metodología propuesta fue capaz de detectar diferencias estadísticamente significativas entre las formulaciones evaluadas (por ejemplo, el fluoruro de estaño fue significativamente más efectivo que los otros tres dentífricos

para *P. gingivalis*, más efectivo que triclosán y que 1,45 de fluoruro de sodio para *F. nucleatum* y que 1,45 de fluoruro de sodio y 2,5 de fluoruro de sodio para *A. actinomycetencomitans*.

Estos efectos antimicrobianos del fluoruro de estaño han sido descritos en otros estudios *in vivo* (Addy et al. 1997), mostrando que el fluoruro de estaño fue superior a los dentífricos de fluoruro convencionales, en términos de inhibición de placa. Además, la eficacia antiplaca y antigingivitis de las pastas de fluoruro de estaño comerciales fue establecida en varios ensayos clínicos aleatorizados de 6 meses de uso en casa (Serrano et al. 2015), lo que lo provee de un alto nivel de evidencia en la evaluación de productos de higiene oral. Algunos de los ensayos clínicos aleatorizados testaron la formulación más reciente, incluyendo SHMP (hexametafosfato de sodio).

Otro hallazgo relevante del presente estudio en relación con las comparaciones entre los dentífricos, fue que 2,5 de fluoruro de sodio resultó significativamente mejor que 1,5 de fluoruro de sodio para *F. nucleatum* y *P. gingivalis*. El fluoruro es el agente activo más importante en los dentífricos, combinado con el cepillado mecánico, en la prevención de la caries dental (Cury y Tenuta 2014). Sus efectos son dependientes de la concentración (Davies et al. 2010), lo que confirma los resultados del presente estudio *in vitro*.

Otro resultado interesante de esta investigación fueron los efectos limitados *in vitro* de triclosán al compararlo con las otras formulaciones testadas, ya que resultó sólo más efectivo que el 1,45 de fluoruro de sodio para *P. gingivalis*. Triclosán es un bisfenol no iónico de baja toxicidad y un agente germicida con un amplio espectro de actividad (contra bacterias Gram-negativas y Gram-positivas) (Lindhe 1990). Al combinarlo con PVM/MA (copolímero of metil vinil eter y ácido maleíco), triclosán puede alcanzar

actividad antimicrobiana durante más de 12 h tras el cepillado. La evidencia científica, basada en su uso en casa, a 6 meses en ensayos clínicos aleatorizados, es suficiente para demostrar los efectos antiplaca y antigingivitis de las pastas con triclosán y PVM/MA (Serrano et al. 2015).

El nivel de concordancia entre los resultados *in vitro* del presente estudio y los resultados de los ensayos clínicos aleatorizados a 6 meses pueden estar asociados a los supuestos efectos antiinflamatorios de la molécula de triclosán (no relevante para este modelo) y la limitada relevancia en estudios *in vitro*, de la presencia del PVM/MA en la formulación.

Los efectos del fluoruro de estaño y de triclosán han sido directamente comparados en un ensayo clínico aleatorizado de 6 meses (Archila et al. 2004) y se encontraron mejores resultados para el fluoruro de estaño comparado con triclosán, en términos de índice gingival y sangrado gingival. Por otra parte, otros estudios clínicos describieron mejores resultados para triclosán en referencia al índice de placa e índice gingival, ambos en estudios de 24 h (Barnes et al. 2010), 6 semanas (Ayad et al. 2010; Singh et al. 2010) o 6 meses (Boneta et al. 2010). Finalmente, otro trabajo no encontró diferencias estadísticamente significativas entre productos, bajo condiciones de no cepillado (Binney et al. 1997).

Las dificultades para evaluar la efectividad de los dentífricos sin la variabilidad asociada al cepillado condujo al propósito de prepararlos en forma de “slurry”, para emplearlos como un enjuague, evitando la necesidad de cepillar. Esta técnica se ha aplicado previamente en diferentes estudios, bajo condiciones de no cepillado **(**Addy et al. 1997; Binney et al. 1997; Moran et al. 2005; Van Strydonck et al. 2006; He et al. 2010), en un estudio *in vitro* (Verkaik et al. 2011) o en un estudio *in vitro* y *ex vivo* (Haraszthy

et al. 2010). Estos estudios testaron las diferentes pastas en forma de "slurry", empleándolos como enjuagues y bajo condiciones de no cepillado. El presente estudio confirma que el método de "slurry" es válido para comparar dentífricos en un modelo de biofilm *in vitro*, incluso comparándolo con otros estudios *in vitro* e *in vivo*, consiguiendo resultados similares. Además, está demostrada la validez de la técnica de qPCR para medir los efectos antimicrobianos de los agentes antiplaca, ya que fue capaz de detectar y cuantificar exactamente las bacterias viables después del tratamiento antimicrobiano. Este método PMA-qPCR fue testado anteriormente en nuestro laboratorio con el modelo propuesto de biofilm *in vitro*, mostrando una clara distinción entre el ADN de células vivas y muertas (Sánchez et al. 2014).

3. Evaluación del impacto en la susceptibilidad del modelo de biofilm a diferentes antimicrobianos en colutorio.

Los resultados de esta investigación mostraron una reducción similar en el número de bacterias vivas de *A. actinomycetencomitans*, *P. gingivalis* y *F. nucleatum* (en CFU/mL), incluidas en un modelo de biofilm *in vitro* formado sobre superficies SLA de titanio y óxido de zirconio, el cual fue expuesto a diferentes agentes antimicrobianos: clorhexidina/cloruro de cetilpiridinio, aceites esenciales y cloruro de cetilpiridinio.

No se encontraron diferencias estadísticamente significativas con respecto al agente antimicrobiano empleado y se obtuvieron resultados similares cuando la aplicación fue puramente química o combinada con agitación.

Se ha reafirmado que, a pesar de que la colonización bacteriana temprana puede estar influida por diferentes características de la superficie del implante, la biopelícula madura es bastante similar en todos los materiales de implante, hallándose un número similar de bacterias vivas en la superficie SLA de titanio y en la de óxido de zirconio

(Sánchez et al. 2014; Schmidlin 2013; Rimondini 2002; de Oliveira 2013; Zhao 2014; de Avila 2015).

El estudio persiguió conocer la eficacia de agentes antimicrobianos sobre biofilms asociados a implantes con diferentes características en su superficie. La información sobre este tema es todavía limitada y aún más escasa si se pretende investigar la posible diferencia en la respuesta de ciertas bacterias incluidas en un biofilm multiespecies formado sobre diferentes superficies de implantes. Hay abundante evidencia científica que demuestra la eficacia de los colutorios antiplaca en enfermedad periodontal y periimplantaria (Graziani et al.2012). En estudios a corto y largo plazo, la clorhexidina resulta más efectiva en reducción de placa que, entre otros, colutorios con aceites esenciales y cloruro de cetilpiridinio. (Felo et al. 1997; Heitz-Mayfield et al. 2011; Porras et al. 2002; Thone-Muhling et al. 2010; Ciancio et al. 1995; Truhlar 2000; Herrera 2013). También, Pan et al. (2010) revelaron en un estudio *in vitro* que en un modelo de biofilm de flujo constante, colutorios que contenían aceites esenciales y clorhexidina mostraron una actividad antimicrobiana similar, pero resultaron más efectivos que formulaciones con cloruro de cetilpiridinio, mientras que en un modelo conocido como “batch chamber slide biofilm model”, el enjuague con aceites esenciales fue significativamente más efectivo que los enjuagues que contenían amina y fluoruro de estaño, una combinación de cloruro de cetilpiridinio/clorhexidina y otro con cloruro de cetilpiridinio. Ready et al. (2015) evaluaron las propiedades “antibiofilm” de dos agentes antimicrobianos (clorhexidina y delmopinol) en un biofilm multiespecies, observando que la clorhexidina al 2% fue el agente más efectivo, consiguiendo una reducción de bacterias totales del 96,2% al 99,99%. Sin embargo, la evidencia en la eficacia de los agentes antimicrobianos en

biofilms maduros asociados a superficies de implantes es limitada y todavía existe controversia en cuanto a si la topografía de la superficie del implante y su tratamiento químico pueden afectar a la capacidad de los agentes antimicrobianos a influir sobre la composición del biofilm y, por lo tanto, a su patogenicidad.

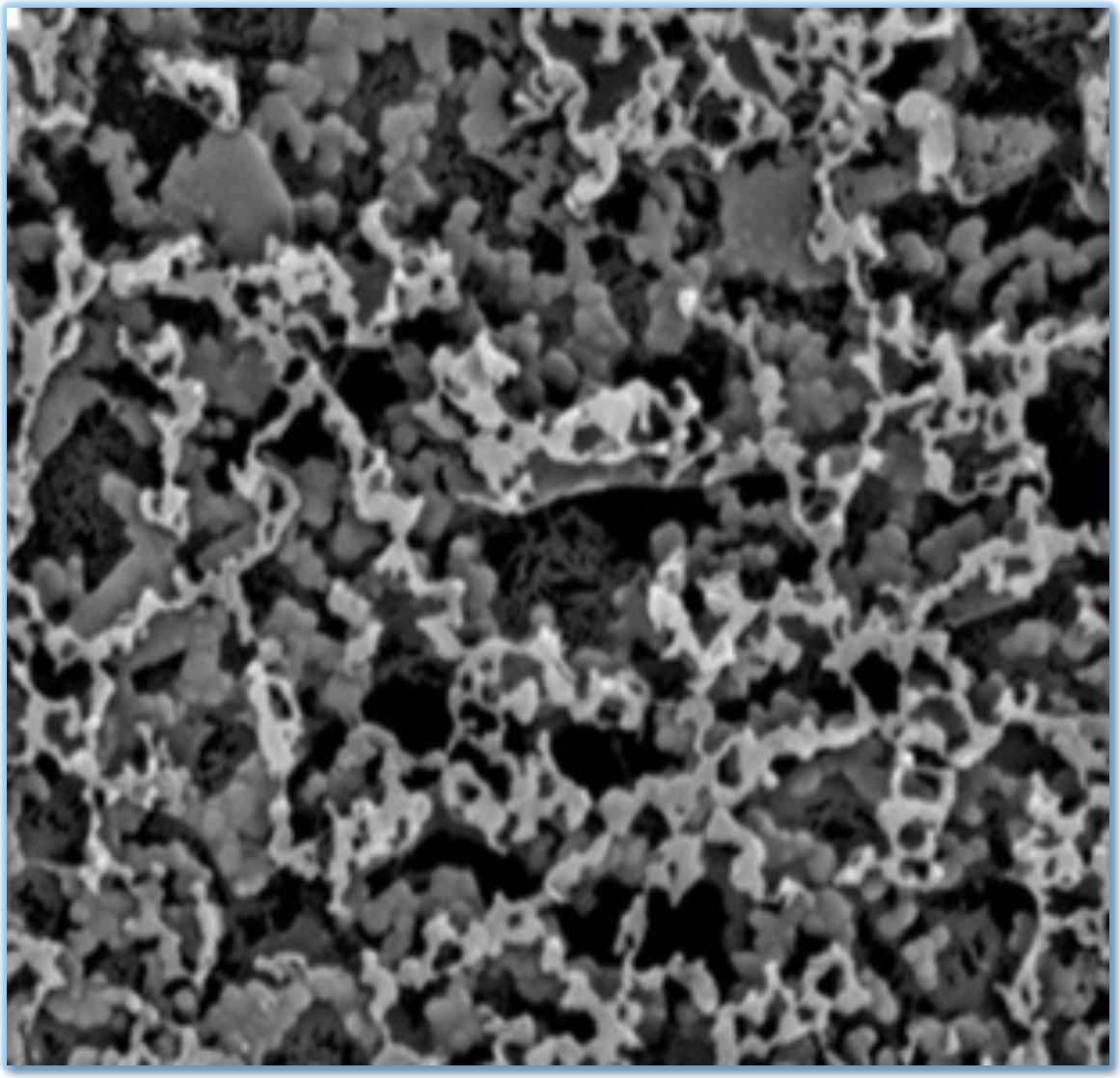
Los resultados de esta investigación muestran el efecto de tres agentes antimicrobianos diferentes (clorhexidina/cloruro de cetilpiridinio, aceites esenciales y cloruro de cetilpiridinio) sobre las bacterias seleccionadas incluidas en el modelo *in vitro* de biofilm formado sobre superficies de titanio (SLA) y óxido de zirconio, las cuales respondieron de forma similar a los agentes antimicrobianos seleccionados, tanto al aplicarlos solos como con agitación. Para las bacterias diana, al comparar el efecto de los agentes antimicrobianos (clorhexidina/cloruro de cetilpiridinio, aceites esenciales y cloruro de cetilpiridinio) con el control negativo (tampón fosfato salino), se muestra una reducción significativa en la vitalidad de las bacterias, siendo la clorhexidina/cloruro de cetilpiridinio la más efectiva, a pesar de que no se detectaron diferencias estadísticamente significativas dependientes del agente antimicrobiano empleado. De manera similar a nuestra investigación, Al- Radha et al. (2013) evaluaron la efectividad *in vitro* de agentes antimicrobianos naturales y gluconato de clorhexidina para reducir el desarrollo del biofilm en superficies de implantes dentales de titanio y zirconio empleando el modelo conocido como “constant depth film fermenter (CDFF)” y encontrando una disminución significativa en la adhesión bacteriana en los primeros dos días tras los tratamientos antimicrobianos en ambas superficies. Al igual que Baffone et al. (2011), que compararon la eficacia en reducción de biofilm periimplantario *in vitro* de clorhexidina al 0,2%, aceites esenciales, fluoruro de estaño y hexetidina asociada con metilparabeno y propilparabeno y concluyeron que la

clorhexidina fue el agente más efectivo. De manera similar, Erriu et al. (2013) obtuvieron efecto antimicrobiano sobre *A. actinomycetencomitans* al tratarla con colutorios libres de alcohol: aceites esenciales y clorhexidina al 0,12%, siendo la clorhexidina, una vez más, el agente más eficaz. Verardi et al. (2016) en un trabajo reciente, estudiaron el potencial efecto de antisépticos como clorhexidina, chloramina T (CHT), triclosán y aceites esenciales sobre la adhesión bacteriana y en la formación del biofilm empleando la técnica de microcosmos, en la que el ambiente de la cavidad oral y las condiciones periodontales se simularon *in vitro* sobre discos de titanio con diferentes tratamientos en su superficie (superficie lisa, superficie lisa al ácido, superficie con chorreado de arena y con chorreado de arena y grabada al ácido). Pudieron observar que los diferentes antisépticos reducían el número de bacterias en los implantes de titanio y, como en nuestro caso, no hallaron diferencias significativas entre clorhexidina y chloramina T o aceites esenciales. Sin embargo, Lin et al. (2013) reportaron que la rugosidad de la superficie influye fuertemente en la eficacia de la clorhexidina sobre los biofilms formados por *S. mutans* y *P. gingivalis* en el modelo de biofilm *in vitro* que emplearon. Una elevada rugosidad resulta en una disminución de su eficacia. Además, la eficacia del tratamiento se reducía significativamente sobre biofilms maduros.

En resumen, mientras que algunos parámetros de la superficie pueden afectar significativamente a la formación del biofilm y a la distribución tridimensional de las bacterias en el biofilm sobre implantes, todavía existe controversia sobre si el tipo de superficie y el tratamiento químico pueden influir en la capacidad de los agentes antimicrobianos para afectar directamente en la vitalidad del biofilm.

Pese a la dificultad que supone intentar reproducir en el laboratorio el ambiente de la cavidad oral y las distintas situaciones clínicas que se pueden dar en ella, el modelo de biofilm *in vitro* empleado en esta serie de estudios ha demostrado su idoneidad para comparar diferentes productos antimicrobianos. Esto es válido tanto sobre superficies de implantes (titanio y óxido de zirconio) como sobre superficies dentarias (hidroxiapatita), lo que lo convierte en un método fiable para evaluar la capacidad antimicrobiana de los distintos agentes antisépticos antes de evaluarlos en ensayos clínicos. El siguiente paso será evaluar el efecto del modelo de biofilm sobre implantes comerciales para tener una visión, todavía más cercana, de lo que ocurre en la cavidad oral; y probar diferentes agentes antimicrobianos, como compuestos de origen natural como los polifenoles del vino, con el fin de evitar los efectos secundarios que presentan los antisépticos como la clorhexidina.

Conclusiones



1. El modelo de biofilm *in vitro* estudiado ha demostrado su validez para el desarrollo de biofilms periimplantarios y para evaluar tanto la influencia de la superficie del implante sobre la formación de biofilm como el efecto comparativo de diferentes agentes antimicrobianos, formulados como colutorios o como "slurry".

2. La estructura tridimensional de los biofilms está condicionada por la superficie sobre la que se forman: el uso combinado de microscopía láser confocal y electrónica de barrido a baja temperatura, junto con la técnica de la reacción en cadena de la polimerasa en modalidad cuantitativa han demostrado su utilidad para el estudio de la distribución espacial del biofilm generado sobre cada uno de los materiales y la viabilidad de los microorganismos en el mismo. Tanto el titanio como el zirconio son igualmente susceptibles para ser colonizados por bacterias orales y de permitir el desarrollo y maduración de biofilms bacterianos.

3. Se ha demostrado la validez del método de "slurry" para comparar el efecto antimicrobiano de diferentes dentífricos sobre un modelo de biofilm multiespecies *in vitro,* empleando técnicas de biología molecular, y se han detectado diferencias significativas en los efectos antimicrobianos de los diferentes dentífricos testados.

4. A pesar de las limitaciones de emplear un modelo *in vitro*, las especies bacterianas *Aggregatibacter actinomycetencomitans*, *Porphyromonas gingivalis* y *Fusobacterium nucleatum*, incluidas en el modelo de biofilm *in vitro* formado sobre superficies de titanio (SLA) y óxido de zirconio, sufrieron un descenso similar en su vitalidad al ser expuestas a los diferentes agentes antimicrobianos, ya fuera en aplicación directa o con agitación.

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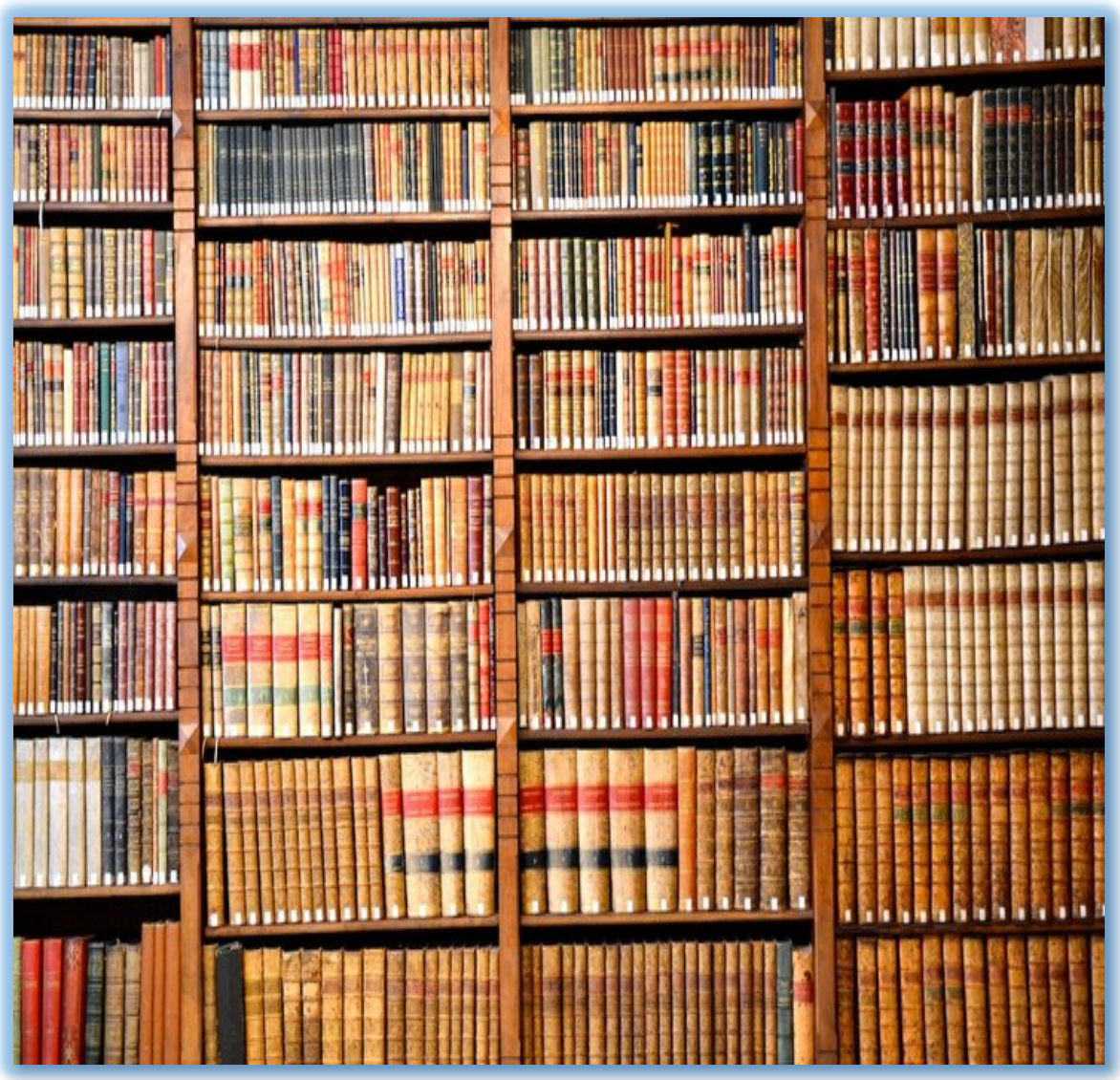

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